METABOLIC ASPECTS OF RENAL FUNCTION

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PREFACE

THE DECISION to bring together these chapters arose in several ways The first of course was a continuing interest in the subject of renal biochemistry an area defined defended and illustrated in the pages that follow Secondly it seemed time to gather together in a systematic way some of the material of this subject in an attempt to examine it critically and evaluate the field both for its present status and future development In doing this it became apparent that like other areas of research this one is not as new as it originally seemed The third and final stimulus arose from the preparation of a review on Kidney Water and Electrolyte Metabolism for the *Annual Review of Physiology* of 1958 The lines of what I wanted to say in a book seemed clear after composing that article

The book that resulted must not be considered in any sense a comprehensive treatise of this large and growing subject Rather it represents a selected series of topics essays or themes which struck me as of particular interest Thus the book has also served as a personal opportunity to draw together and place in a larger context much of the research with which I have been concerned over the last fifteen years This is particularly true in the area of phosphate and amino acid transport ammonia excretion the mechanism of action of phlorizin the relation between renal excretion and utilization of metabolic substrates and the role of the tricarboxylic acid cycle in the kidney I have not worked directly in the field of organic acids and bases thus in the chapter on this subject I have had to tell a story as an outsider It was included because it is a fascinating trail of research full of frustrations and brilliant strides and in addition it has never been brought together before in its consecutive development

The important field of urinary acid secretion carbonic anhydrase and its inhibitors the chlorothiazide development and

the problem of the mercurials has not been included The decision to do this was taken after some thought These are subjects that others in the field are eminently better qualified to handle and have indeed done so in several excellent critical reviews The reader is referred particularly to the article by Pitts entitled Some Reflections on Mechanisms of Action of Diuretics in the Symposium on Renal Physiology in the May 1958 issue of the *American Journal of Medicine*

In all that follows the work of many investigators past and present is woven into the story It is hoped that neither names nor work are anywhere taken in vain If there are misrepresentations of ideas or data the responsibility is solely mine

Throughout these essays there is more or less speculation Such is an author s perogative in this kind of book and indeed it is often such speculation that can lead to fruitful new concepts which can only be placed in a more solid frame by careful experimentation At points ideas for such experiments are thrown out These are not offered because they claim to be brilliant but rather because they occurred in the context of thought about *that particular subject and thus seemed naturally to belong in* its discussion

Whether a unitary theme is apparent in these essays will only be clear to the individual reader In concept the main theme has been the relation between cell metabolism and the specialized functions that characterize the kidney As mentioned in the introduction the emphasis has been placed on metabolites in the broadest use of this term rather than on water and electrolytes Thus one of the themes that is meant to appear throughout is the relation between the renal assimilation of metabolites and their mechanism of transport This is the primary reason for choosing to discuss phosphate amino acids glucose and the organic acids of the tricarboxylic acid cycle In this same vein ammonia by virtue of its intracellular origin from amino acids and their amides may be considered a metabolite

In addition the glycoside phlorizin deserves special consideration as a unit in this theme It is classically associated with glucose transport across certain cells Thus to understand the

mechanism of action of phlorizin may mean to understand the biological transport of glucose In addition, the phlorizin story illustrates another theme that recurs throughout the book, that is, the increasingly apparent fact that active transport mechanisms are coming to be understood more as membrane phenomena than in terms of the reactions supplying them energy

The task of committing these chapters to paper was made pleasanter and much more stimulating by an opportunity to present them as lectures at the Mount Desert Island Biological Laboratory, Salisbury Cove, Maine, during the summer of 1958 The atmosphere there was conducive to quiet thought, stimulating discussion, and helpful criticism from many friends

Much of the work included here from the author s laboratory was done in collaboration with a group of colleagues, the association with whom has been a continually rewarding experience In addition, a special debt of gratitude goes to the two men, Dr Robert F Pitts and Sir Rudolph A Peters F R S, who have done so much to provide a backlog of ideas and shape attitudes towards research Thanks also go to Dr Homer W Smith for advice, criticism and the invitation to lecture on these topics at the Salisbury Cove laboratory And lastly it is a pleasure to acknowledge the excellent art work of Mrs Margaret Cook and Professor Joseph Homan of the Department of Medical Arts for the preparation of the charts and to express thanks to Mrs Barbara Wold for invaluable bibliographic and secretarial work in preparation of the manuscript

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1

INTRODUCTION

FOR MANY YEARS the kidney was regarded as a purely excretory organ, sieving waste products from the blood However, for several reasons this idea did not last into the present century Arthur Cushny with his Modern View pictured renal function of a more discriminating sort For besides its excretory activities he stressed the role of the kidney in the conservation and regulation of body fluids Not only were no threshold substances excreted and threshold substances retained, but in addition Cushny pointed out that a fluid of optimum composition is reabsorbed by the tubules and this in its resemblance to extracellular fluid serves remarkably well to maintain the integrity of that compartment This *regulatory* function has been stressed since Cushny in the work of L J Henderson, Gamble, Van Slyke, Smith Pitts and others and it is this aspect of renal physiology which demands our attention today We must therefore, take a broader look at the role of this remarkable organ in the overall economy of the body

The work of the last fifty years or so has furnished us with a more precise description of the several aspects of the excretory process as it occurs in each of the two million or so nephrons which make up the two kidneys The formation of an ultrafiltrate of plasma in the capsular space from glomerular capillary blood has been proven beyond doubt by the micropuncture studies in A N Richard s laboratory These studies also provided a direct demonstration of tubular reabsorption and secretion, the first postulated in Cushny s theory and the second, demonstrated indirectly by Marshall and Vickers work with phenol red

In addition, the micropuncture work clearly demonstrated the acidification of the urine and the secretion of ammonia in the

same discrete segment of the distal tubule These are two of the important synthetic activities of the kidney, they play a central role in the regulation of acid base balance, and they present us with two of the most intriguing aspects of renal biochemistry

The quantitative aspects of renal function in higher animals and man have been extensively studied in the last twenty years with the clearance techniques introduced by Rehberg and Van Slyke and so beautifully developed by Homer Smith and his colleagues who saw and exploited the full potentialities of the method

From this work and its subsequent development there have emerged three main areas which appear to be the most active and pressing in contemporary renal physiology These are 1) the general problem of water and osmoregulation 2) the regulation of renal blood flow, and 3) the biochemical aspects of renal function Although perhaps of no greater importance than the other two, it is with the last of these three subjects that we will be concerned in this book Here in the introduction, therefore, we must define what we mean by these biochemical aspects of renal function

All cells of the body exhibit common features of a general biochemical architecture, among these are glycolysis, aerobic oxidation, and protein synthesis But adapations of these general functions as well as special reactions of their own (like hippuric acid formation) characterize the cell chemistry of different organs Thus to understand fully the role of the kidney in the general physiology of the body, we must study both its general and its special functions A comparison with the liver will emphasize this point The liver excretes bile as the kidney excretes urine, and in many ways the two processes are similar But to see the liver solely in terms of its biliary function would be an extremely myopic view of an organ with its host of other important metabolic activities In like manner the kidney must be looked at as an organ with an importance beyond its purely excretory role It is this area in the kidney that may be termed the 'metabolic or biochemical' aspects of renal function

One might justifiably ask why an entire book need be devoted to these aspects of kidney physiology Are they of such

special interest or importance that they warrant such an outflow of words and effort? For several reasons the answers to these questions are affirmative By way of introduction we may look at some of them in detail, and to begin it seems logical to examine the kidney from the standpoint of its overall energy metabolism

The kidneys exist under a high oxygen tension, they receive one fourth to one third of the total cardiac output at essentially aortic pressure Any one who has looked at renal venous blood is impressed with its arterial nature Indeed, as this would indicate, the amount of oxygen released by each milliliter of arterial blood as it traverses the kidney is small in comparison to some other organs, but because of the tremendous blood flow to the kidneys their total oxygen extraction is actually greater than any other organ in the body

In muscle the oxygen requirements for external work are obvious, and therefore, the reason for a high coefficient of oxygen absorption in these tissues is readily understandable In the kidney, however, we shall see that no such apparent relationship exists between oxygen consumption and external work, and this fact poses one of the most mysterious problems in renal physiology Let us examine this statement in greater detail

In 1905 von Rhorer[1] derived the equation for calculating the osmotic work done in the formation of urine The total work was taken as the sum of the partial osmotic works in concentrating or diluting each of the constituents of the plasma A number of investigators have measured osmotic work during a variety of experimentally produced conditions such as urea, sulfate, or mannitol diuresis and saturation of the tubular transport mechanism for p aminohippurate In most of these studies renal oxygen consumption, and sometimes heat production, was measured

A number of earlier studies, such as those of Barcroft and Brodie,[2] Barcroft and Straub,[3] and Knowlton and Silverman,[4] made it appear that osmotic diuresis, as during sulfate infusion, is accompanied by increased oxygen consumption by the kidney However, Fee[5] later showed that if renal blood flow were kept constant, there was no increase in renal oxygen consumption under these circumstances In a study of the ' Gaseous Metabolism of the Dog's Kidney in 1928, Hayman and Schmidt[6] reached

similar conclusions They were unable to find any effect of caffeine, urea, or sodium sulfate on renal oxygen consumption They noted that the coefficient of oxygen utilization is smaller at higher renal blood flows than with lower, but nevertheless the total oxygen consumption of the kidney goes up with rising blood flow Indeed, Hayman and Schmidt showed that oxygen consumption and blood flow in the kidney are directly related phenomena In a later paper Van Slyke, Rhoads, Hiller, and Alving[7] showed in the unanesthetized dog with one explanted kidney this same relation between renal oxygen consumption and renal blood flow These investigators believed that whatever metabolic requirement alters the one function likewise alters the other Van Slyke *et al* demonstrated what Hayman and Schmidt had also observed, namely, that the increased osmotic work required of the *kidney in excreting an imposed load of urea is performed with*out any measurable change in oxygen consumption

A study some four years later in 1940 by Eggleton, Pappenheimer, and Winton[8] reached similar conclusion from experiments on the anesthetized dog In these experiments renal oxygen consumption was continuously monitored by photoelectric means, and renal osmotic work was calculated from plasma and urine measurements of several crucial solutes Using a similar system, Kramer and Winton[9] in a study of the influence of urea and changing arterial pressure on oxygen consumption again reached a similar conclusion when they found that urine formation and oxygen consumption by the kidney are not related phenomena

Barcroft and Brodie[2] and Glaser, Laszlo, and Schürmeyer[10] have pointed out that the mechanical work performed by the kidneys in concentrating and excreting the urinary solutes constitutes less than one per cent of the total renal energy production as measured by its oxygen consumption Recalling this fact, Van Slyke states that The overwhelmingly greater part of the energy produced must be utilized by the kidney for its own internal cellular processes not related to the external work which the organ is performing In this respect the kidney affords a contrast to muscle '[7]

Usually when we speak of the mechanical efficiency of a machine or an animal we refer to the ratio between external

work performed and total energy produced as measured by oxygen consumption or heat production In their study of the isolated dog kidney, Eggleton, Pappenheimer, and Winton[8] discussed the efficiency of the kidney in performing osmotic work in these same terms However, in a sense to do so seems somewhat unjust Because if the bulk of the renal energy production does, indeed, support *non-excretory functions, which* may be just as important in the physiologic contribution of the organ then an increase in the osmotic work without a concomitant increase in oxygen consumption can hardly be regarded as more 'efficient ' Such a view assumes that the non excretory energy expenditure belongs to an inferior category like the heat lost by an inefficient machine Therefore, until we understand the nature of this 99 per cent of renal energy going for non-excretory purposes it would seem better not to relate it to measurable work in terms of efficiency

Be that as it may, an important point of this paper needs stressing Eggleton *et al*[8] showed that an increased osmotic work of around 38 per cent (during a pressure diuresis) was associated with an increased renal efficiency of 25 per cent Similarly in a urea diuresis the increase in osmotic work accompanying a doubled urine flow was in the order of 63 per cent, and the organ s efficiency rose under these circumstances by about 59 per cent It must be stressed, however, that the total efficiency, though increased in these terms, never exceeds a few per cent. To express it in *these terms makes more striking the fact that renal* work changes are accomplished without measurable alterations in renal oxygen consumption so long as blood flow remains constant And under these conditions all attempts to show changes in oxygen consumption during forced excretory loads have failed[5,6,7,11]

A recent paper by Crosley *et al*[12] would appear at first sight to represent an exception to this conclusion It reports an increase in renal oxygen consumption during the infusion of sodium lactate in man This study was carried out on six patients all of whom had renal disease For several reasons, however, the conclusions reached in this work must be regarded with some caution For instance, in two of the six cases (33 per cent of the series) the increased oxygen consumption was 5 and 8 per cent Of the

others only two cases showed an increase of as much as 50 per cent in the remaining two it was 25 and 15 In terms of actual change some of the differences fall very close to the limits of accuracy of the Van Slyke gasometric method for blood oxygen Therefore, while representing the only reported change in renal oxygen consumption without a change in renal blood flow, this paper must remain of interest, especially because of the lactate infusion However, one would want to see greater differences in a larger number of cases before accepting the findings unequivocally

If the extremes of external excretory work are accomplished by the kidney without measurable changes in total oxygen consumption, one might legitimately ask why the kidney exists under such a high oxygen tension Perhaps its enzyme systems require a high oxygen tension for optimal activity

Although it is known that the kidney can synthesize and break down glycogen, the organ does not display a pattern of glycolytic metabolism in any sense comparable to that of liver or muscle Its metabolic pattern appears to be predominantly aerobic in contrast to the capacities for anerobic energy metabolism in these latter two tissues In addition the Q_{O_2} of kidney tissue in air or oxygen is about twice as high as liver Kidney possesses a very active tricarboxylic acid cycle, again in contrast to liver where this system is less active Furthermore, as Bayliss and Lundsgaard[13] showed in 1932, the kidney perfused with cyanide loses in large measure it ability to concentrate the urine and thus to perform the osmotic work we have been discussing For these several reasons one would suspect that the capacity of the kidney to carry out osmotic work must depend primarily on aerobic energy metabolism

To test this hypothesis more osmotic work experiments should be done under relatively anaerobic conditions such as breathing low oxygen mixtures or injection of agents that form methemoglobin Such studies would however, have to be interpreted in the light of Pappenheimer and Kinter's cell skimming hypothesis [14] for according to this scheme the tubules would be supplied with blood relatively poor in erythrocytes But these studies would have the advantage over cyanide experiments in

that cellular enzyme systems would be essentially intact during their function at reduced oxygen tension

From the foregoing discussion it is apparent that we must turn to more discrete studies of renal intermediary metabolism in a search for the subtle biochemical reactions responsible for the energy supply for renal work The energy requirements of ion and water movements and the maintenance of gradients in these functions must be more closely studied Turnover studies with isotopically labelled substrates, enzymes, and coenzymes will also be required Studies already begun[15,16] on the simultaneous extraction and excretion of metabolites and the identification of their end products must be extended to include a variety of altered excretory "conditions" In addition, valuable information may accrue from a continuation of combined correlative studies with several experimental conditions that affect both renal tissue *in vitro* and intact renal function *in vivo* Discussions of this sort of work will appear in subsequent chapters but still the mystery remains, we simply cannot account at present for the "use" made by the kidney of 99 per cent of its energy production

As yet little is known of the role of the kidney in the general metabolism of protein, carbohydrate, and fat It is known that the kidney produces glucose[17] and is capable of gluconeogenesis[18] and glycogenesis[19,20] The glycogen is synthesized from glucose, but its function in the tubular cells is not known As mentioned earlier, it is present in much smaller amounts than normally seen in liver Marsh and Miller[19] estimate the renal glycogen at approximately 0 04 per cent It is hard to visualize the kidney playing a quantitative role in blood sugar regulation in any way analogous to that of the liver, and the normally small quantities present in kidney would underline this thought

One other observation of renal carbohydrate metabolism is of interest Before the advent of insulin many cases of diabetic death showed glycogen nephrosis at autopsy Robbins[21] believed this must mean that renal glycogen level is a function of blood sugar concentration as it responds to insulin He performed experiments with rats that seem to corroborate this view In alloxan diabetes glycogen nephrosis appears, then disappears with insulin treatment The deposition of glycogen in the tubular epithe

lium starts at a blood glucose level around 350 mg per cent The 'physiologic' nature of this change is supported by the fact that the tubular epithelium appears perfectly normal after the glycogen deposits have disappeared With the widespread use of insulin in diabetes, glycogen deposits in the renal tubule cells are no longer seen so frequently at autopsy

The role of the kidney in the protein economy of the body is also an unknown factor Except for the synthesis of angiotonin nothing is known about protein production by the kidney for use elsewhere in the body In its angiotonin production the kidney seems almost like an endocrine organ Perhaps other similar functions will come to light

It goes without saying however, that the kidney has a rapid and active protein metabolism of its own This fact is emphasized by the rapid renal hypertrophy that occurs after unilateral nephrectomy In the intact animal the protein turnover in the kidney is as rapid as several other organs and apparently more so than liver For example, Schoenheimer[22] measured the N^{15} content of protein nitrogen from different organs after feeding N^{15} labelled L( ) leucine and glycine After leucine, kidney was as rich in N^{15} as intestinal wall, almost as rich as serum, and one and one half times as much as liver Whether this dynamic state in its protein pool has functional implications beyond the internal economy of the kidney cannot be said in the present state of our knowledge One wonders, however, whether a general phenomenon resulting from renal malfunction, such as the anemia of renal insufficiency, will ultimately be explained in some such terms

What relation the tubular absorption of protein bears to these several aspects of renal protein metabolism likewise cannot be said Oliver[?2] has clearly and repeatedly observed the intracellular protein droplets that appear in the proximal tubular cells within several hours after the intraperitoneal injection of egg white protein These droplets have been shown by immunologic means to consist of the same egg white protein that was injected Their appearance and disappearance, after a variable time, coincides with the dissolution and ultimate reconstitution of the intracellular mitochondria The final disposition of the egg

white protein that is filtered and then absorbed by proximal tubule cells is not apparent from Oliver s studies However the active participation of the cell mitochondria indicates that the foreign protein has somehow been assimilated or turned over in the cell and thus entered its protein mass Clearly more work is needed before these and many related problems in renal protein metabolism are answered

We have emphasized above the fact that the reasons for the bulk of the active aerobic metabolism of the kidney are largely unknown We shall see however in a later discussion that the tricarboxylic acid cycle plays a synthetic and regulatory role in the kidney beyond its usual one as the final common pathway of aerobic oxidation Several of the organic acids that comprise this cycle are excreted in the urine in increased amounts under various circumstances and appear to play a role in anion conservation and in calcium excretion In addition recent experiments indicate that certain segments at least of this cycle are involved in the reabsorption and tubular secretion of some of the cycle s own intermediate substrates Here a whole new area of renal metabolism has opened up

In the renal regulation of acid base balance there is a wealth of information that rightly belongs to the subject of this book We have chosen to deal specifically with the problem of ammonia production and secretion This process is closely allied with the secretion of hydrogen ions and both mechanisms constitute the means for conserving fixed cation in the kidney The reactions of ammonia synthesis in tubular cells in the distal segment are intimately tied up with both amino acid metabolism and the tricarboxylic acid cycle It appears that urinary ammonia is derived from plasma amino acids and certain of their amides and in turn these reactions are in close and dynamic equilibrium with the cycle of aerobic oxidation

Any discussion of the biochemistry of the kidney must as one of its central themes consider the subject of transport mechanisms A number of important books have appeared on this gen*eral subject*[24, 5] *Our discussions here* are not intended to duplicate these more complete works rather we will concentrate primarily on the transport of metabolites because in several cases

certain reactions of the intermediary metabolism of these substances appear to have been adapted to function (in a way unrelated to these intermediary reactions) as the carrier in their tubular transport mechanism For example phosphate transport appears in some cells at least to involve adenosine triphosphate, and amino acid transport utilizes some of the same reactions with pyridoxal that occur in transaminations within the cell The identification of carriers and the nature of their reactions with the substrate is thus a major aspect of the topic under study

The biochemistry of transport must also be considered from another standpoint The tubular excretion of certain organic acids and bases of exogenous origin has been extensively studied and is rightly a part of our subject This is particularly true of certain dyes and the benzoic and hippuric acid derivatives Experiments with these compounds have taught us much both positive and negative about their mechanisms of transport More over it appears from this work that our study of transport should concentrate more on the cell membrane than on the energy re actions in the cell Intracellular reactions appear less often helpful to our understanding of these mechanisms than do the nature of the substrate-carrier reactions at or in the cell membrane This may seem an obvious statement but much work has focused on the intracellular reactions furnishing the energy for trans port and this has led often to erroneous conclusions concern ing the nature of intracellular energy metabolism in transport mechanisms

Often the use of inhibitors has led in this direction and in no case has this been more evident at some stages than in the study of phlorizin an account of which is given in Chapter VII Although this glycosuric agent does inhibit energy metabolism this effect appears to be secondary to its primary site of action at the membrane Phlorizin has occupied an interesting and important position in renal physiology Not only does it produce an unexplained glycosuria but its use in this regard has played a major role in validating the clearance technique of measuring glomerular filtration rate For the purposes of this book we seek its mechanisms of action in order to understand the biological transport of sugars This study has made good progress and its

implications appear to reach beyond the kidney and even to touch the important problem of insulin

The biochemistry of the kidney forms the basis for the rational development of drugs designed to have a highly specific effect on that organ Witness in this regard the now outmoded use of p aminohippuric acid to block the tubular excretion of penicillin This led to the subsequent development of carinamide and Benemid in Beyer s laboratory Or look at the development of acetazoleamide (Diamox) as a specific inhibitor of carbonic anhydrase work that grew out of the original studies on the effects of sulfonamides on this enzyme[26] When it was shown that sulfanilamide would alkalinize the urine and that this effect could be understood in terms of an inhibition of carbonic anhydrase the search was on for more effective agents to accomplish this effect Acetazoleamide resulted and others followed Now chlorothiazide with some characteristics of a carbonic anhydrase inhibitor appears to accomplish cation loss more perfectly Thus the understanding of a fundamental cellular mechanism leads to important drugs designed to create a specific therapeutic effect

Finally because of the elegant methods for studying its function the kidney presents the physiologist with a unique subject for the general study of cell biology Thus the metabolic aspects of renal function both draws from and contributes to this larger area of knowledge And with this sort of *raison d etre* before us we can see that our subject matter is neither too narrow to merit book length treatment nor too parochial to speak for a broader field of physiology

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II

THE TRANSPORT AND METABOLISM OF PHOSPHATE

INORGANIC orthophosphate is unique among the anions that are transported across cells because of the major role it plays in their intermediary metabolism Coupled with cellular respiration is the formation of a number of purine and pyrimidine nucleotides as well as creatine phosphate The adenine nucleotides in partic ular (adenosine triphosphate ATP —diphosphate, ADP, and—monophosphate, AMP) are central components of cellular energy metabolism More recently other nucleotides such as those of uridine, guanidine and cytidine have also been implicated in important biochemical reactions Certain of the phosphate bonds in these nucleotides contain a rich store of potential energy, and their enzymatic cleavage or transfer releases this energy for use in biological work such as muscular contraction secretion trans port a variety of synthetic reactions, bioluminescence, and photo-synthesis

Much work has been done on the biological transport of phos phate across a wide variety of cells both *in vitro* and *in vivo*, and in a striking number of instances the evidence seems to implicate certain aspects of this metabolism of phosphate in its biological transport It is the purpose of this chapter to examine this evi dence in some detail and discuss its relevance to our understand ing of the tubular transport of phosphate in the kidney

The Characteristics of Phosphate Transport Across the Renal Tubule

In man[1] and dog[2] the rate of phosphate reabsorption in creases directly with its rate of glomerular filtration up to a point

where a maximal rate of transport (Tm PO_4) is reached. At filtered phosphate loads above this Tm the rate of transport remains constant and excess phosphate is quantitatively excreted in the urine. These relationships between quantities of phosphate filtered, excreted, and reabsorbed are illustrated in Figure 1.

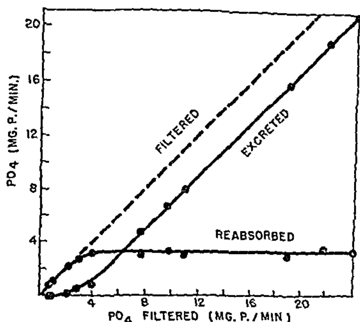
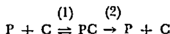


Figure 1. Relation between rates of filtration, excretion, and reabsorption of inorganic orthophosphate in the dog kidney. From Pitts and Alexander (2).

The transport of phosphate, as illustrated in these data, is "active;" that is to say, it is carried out in an "uphill" fashion against an electrochemical gradient. A variety of other substances such as glucose, sulfate, ascorbic acid, and certain amino acids are similarly transported by active mechanisms. In his analysis of such a mechanism in the case of glucose, Shannon[3] postulated the existence of a membrane or cytoplasmic "carrier" of limited quantity or capacity and showed that the maximal rate of glucose transport was the algebraic sum of the several reaction rates of the components of this carrier system. In simplified form, Shan-

non visualized such a transport system requiring at least two overall reactions



$$P + C \overset{(1)}{\rightleftharpoons} PC \overset{(2)}{\rightarrow} P + C$$

As applied to the present discussion, P represents phosphate, C, the carrier present in fixed quantity, perhaps at the luminal border, PC, the carrier phosphate complex Dissociation of the complex liberates inorganic phosphate into the interstitial fluid where it then reaches the peritubular capillary blood Once dissociated, C is then free to combine with more phosphate coming to it from the tubular urine

The shape of the reabsorptive curve in Figure 1, the existence of a Tm, and the magnitude of the Tm obviously depend on a number of variables in such a hypothetical system Among these are The quantity of the carrier substance, the affinity of phos phate for the carrier and the relative velocity constants of the reactions of association and dissociation For there to be a maxi mal transport rate (Tm) the second reaction must be first order with a rate that is slow in comparison to that of the initial one between phosphate and carrier In the cat[4] where no Tm for phosphate has been demonstrated, the elements of the carrier system may be the same as those in dog and man except the first reaction may be slower than the second or at physiologically at tainable phosphate loads the carrier never becomes saturated. Wilbrandt[5] has presented theoretical reasons for accepting the idea that the carrier is in the membrane in the case of glucose, and we will reach the same conclusion for phosphate

Thus an understanding of phosphate transport (as in other similar mechanisms) involves the identification of the carrier,' an estimation of its concentration," its location (on the mem brane or in the cytoplasm), the nature of its reaction with phos phate, and the kinetics of association and dissociation of carrier phosphate complex To say this, of course, is to outline an extremely comprehensive problem, but nevertheless it would appear that nothing less is required for a complete description of the system

It has seemed to the author that a careful examination of the phenomenon of phosphate transport across cell membranes of a variety of living species would be useful in devising a hypothetical approach to the same transport system in higher animals For this reason it is of interest ot examine some of this knowledge

The Comparative Physiology of Phosphate Transport

A number of separate studies in recent years on different biological systems have all tended to reach certain similar con clusions namely, that phosphate in its transport across a variety of cell membranes combines chemically with a membrane carrier And it seems likely that this carrier is in some instances the same adenine nucleotide system discussed earlier, functioning separately from its usual role in intermediary metabolism We shall now examine some of the studies supporting this idea and then pro ceed to a consideration of its relevance to the problem of renal *transport of phospate*

Erythrocytes—When orthophosphate labelled with P^{32} is in cubated with whole blood the phosphate leaves the plasma and enters the cells This phenomenon has been studied by Gourley[6] and exhibits the characteristics of a first order reaction In human blood the per cent P^{32} in the cells increases curvilinearly with time, reaching a constant value by 7 to 8 hours Several character istics of this process show quite clearly that it is not a simple *diffusion of phosphate into the cell* but an active mechanism re-lying in non nucleated cells, largely on glycolysis for its energy Thus the uptake is independent of external phosphate concentra tion dependent on temperature and glucose oxidation and inhibited by iodoacetic acid and fluoride

In an attempt to determine the nature of the chemical reac tions between orthophosphate and carrier, Gourley made tri chloracetic acid extracts of plasma and cells at different time intervals after addition of P^{32} and determined its relative specific activity in the plasma and the several organic phosphorous frac tions of the cell The results of his studies are shown in Figure 2 These data were analyzed by Gourley in terms of the postulates set down by Zilversmit and his associates[7] governing the condi

tions that must be met in steady state situations where one substance is acting as precursor for another The curve of specific activity in the precursor must be initially higher than the one for the product and the two curves must come into equilibrium when the specific activity of the product has reached its maximum

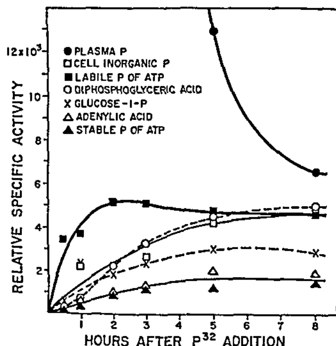


Figure 2 Precursor product study showing relative specific activity of P 32 in plasma phosphorus cell inorganic phosphorous and several organic phos phorous fractions of the red cell at intervals following addition of P 32 to blood. The P 32 appears most rapidly in the labile P of ATP and then equilibrates with the cell inorganic P See text for explanation From Gourley (6)

Looked at in this light, it appears from the data of Figure 2 that the labile phosphate groups of ATP are acting as the precursor of the cell inorganic phosphate and thus that ATP is somehow in volved in the transfer of inorganic phosphate into the cell In addition to experiments such as these, Gourley has shown that

iodoacetic acid and fluoride which inhibit phosphate transport also reduce the concentration of ATP in the red cell and the turnover of P^{32} in both stable and labile phosphate groups of ATP

On the basis of these studies Gourley has suggested that ATP may be the form in which inorganic phosphate is transported across the membrane into the erythrocyte One would have to imagine the ATP in the cell membrane in a state of thermal agitation coming into contact with both the external phosphate in the medium and that inside the cell The scheme would thus visualize phosphate transport in terms of the Shannon hypothesis discussed above with the adenine nucleotide alternately being phosphorylated to ATP and dephosphorylated to ADP Here the adenine nucleotide would be operating as the carrier completely independent of its functions in respiratory energy metabolism As a carrier we may designate it ATP ADP

Sea urchin egg—Similar conclusions have been reached concerning the transport of phosphate by the sea urchin egg In an ingenious study Lindberg[8] showed that the unfertilized sea urchin egg has the capacity to metabolize radioactive orthophosphate in its surface to ATP This metabolizing layer he calculated to be 0 02 to 0 05 microns thick If the fertilized egg is cultivated in sea water to which P^{3} is added it takes up the isotope primarily in the form of ATP which slowly makes its way into the egg This penetrating ATP is largely bound to the structure of the egg and Lindberg notes that it plays no noteworthy part in the egg s metabolism

Cardiac and skeletal muscle—Both skeletal and cardiac muscle have been shown to accumulate inorganic phosphate in a similar fashion Sacks and Altschuler[9] in 1942 studied this phenomenon *in vivo* with radioactive phosphorous and showed that phosphate enters the cells of both these muscle types by being converted to an organic compound presumably at the membrane Conversely phosphate can leave the cell only by the hydrolysis of these organic compounds at the membrane These observations added further support to the earlier work of Furchgott and Shorr[10]

who studied the distribution of P^{32} in organic phosphorous fractions of cardiac muscle slices respiring in the presence of P^{32} *in vitro* They found that the intracellular inorganic phosphate and the phosphocreatine of the cardiac muscle have the same P^{32} content at a level only one fifth that of the medium Had the phosphate entered the cell by simple diffusion they reasoned that the equilibrium P^{32} content would have been the same in both intracellular and extracellular phases

Bacteria—Mitchell and colleagues in a beautiful series of studies[11 12 13] have made a careful analysis of the transport of phosphate across the cell membrane of *Micrococcus pyogenes* At the surface of this cell there is an osmotic barrier impermeable to inorganic phosphate This barrier encloses a volume of some 1 7 ml per gram dry weight of cells and within it the inorganic phosphate of the cell is confined at a concentration of approximately 0 1 M The inorganic phosphate of external and internal media exchange by an active process that is sensitive to phenyl Hg^{+}, arsenate, 2,4-dinitrophenol, aureomycinate, and certain sulfhydryl inhibitors The existence of this osmotic barrier and the characteristics of phosphate exchange across it are in accord with an exchange-diffusion system in which phosphate is shuttled across by carrier molecules Since inhibition of the mechanism does not depend on any alteration of either residual metabolism or fermentation it would seem that the phosphate exchange mechanism although active, depends upon a carrier working within the osmotic membrane barrier relatively independent of the aerobic and anaerobic metabolism of the cell interior This system in *Micrococcus pyogenes* appears to be specific for $H_2PO_4^-$ ion Interestingly, however it can also transport arsenate which competes with a lower affinity than phosphate for the carrier Although not as definite in his conclusion about the nature of this carrier as was Gourley from his work on erythrocytes, Mitchell seems to suspect that the phosphate exchange in bacteria likewise involves organic phosphate combination Perhaps these reactions are similar to the ATP ADP phosphate carrier in the erythrocyte membrane or perhaps they involve some different form of organic phosphorous

combination This substance might then be in the glycero phos phoprotein complex that Mitchell and Moyle[14] had shown earlier to be present in the cell envelope of *Micrococcus pyogenes* and to account for some one quarter of its total organic phosphate

Yeast—Again similar conclusions about the nature of phos phate transport have been reached by Kamen and Spiegelman[15] in their elegant studies with yeast The uptake of phosphate by this organism has a temperature coefficient too large to be based on simple diffusion The process is inhibited by several agents known to block organic phosphate formation, and again there exists the interesting competitive interrelation between arsenate and phos phate for the transport system Exchange is reduced in cells metabolizing slowly, while pre treatment of yeast cells with a substrate in the absence of phosphate accelerates the subsequent phosphate uptake when the cells are transferred to a phosphate medium

The phosphorylation of ADP or some other organic compound in the membrane of the cell during phosphate transport would assume the necessity of the breakdown of the phosphate ester so formed to liberate inorganic phosphate into the cell once trans ported across the membrane A variety of phosphatases have been shown to be present in kidney tubule cells [16] Were the membrane carrier for phosphate the ATP ADP system, this phosphatase would specifically have to be an ATPase That this enzyme is present in at least one cell membrane has been shown for yeast by Rothstein and Meier [17] Kidney contains ATPase, but the writer is not aware whether it is in the cell membrane as well as in the mitochondria

Thus the above described studies have shown a membrane carrier for phosphate in yeast, certain bacteria, erythrocytes, card iac muscle, skeletal muscle, and the developing sea urchin egg Much of the work has involved studies of P^{32} uptake and turn over in the various organic phosphorous fractions of these cells, and all of it points to some form of organic phosphate combination in the membrane as a component reaction of the phosphate carrier system

Situations Affecting Phosphate Metabolism and Transport in Kidney

We will now turn our attention back to the renal tubular transport of orthophosphate in mammals examine certain situa tions where the transport is altered *in vivo* and where these same situations have been shown to be associated with a change in phos phorous metabolism in the kidney *in vitro* These instances when coupled with the evidence cited above lead one to the hypothesis that phosphate transport across tubular cells of higher forms may likewise involve the formation of organic phosphate complexes at the cell membrane

A *Parathyroid*—It has long been known that disturbances of parathyroid function are associated with altered blood levels and urinary excretion of phosphate It is of particular interest to the present thesis that the injection of parathyroid extract is associated with a phosphaturia in several species including man [18] dog [19] and rat [0] Indeed Tepperman L Heureux and Wilhelmi[21] have taken advantage of this phosphaturia in developing an assay procedure for parathyroid hormone We will not be concerned here with the various fractions of parathyroid extracts and the separate effects of these on calcium and phosphorous in the body Rather we will start with the conclusion (now rather well founded) that parathyroid hormone does have an effect on phos phate excretion in the kidney and examine the nature of that effect in the light of the hypothesis under consideration namely that phosphate transport may involve some of the elements of cellular phosphate metabolism

Several studies have shown [18 22] that the single injection of parathyroid extract is sometimes associated with elevated rates of glomerular filtration and renal blood flow and that the attendant phosphaturia may merely result from the accompanying elevation in filtered load of phosphate with no demonstrable alteration in tubular reabsorptive capacity The possibility of a specific tubular effect of parathyroid hormone on phosphate reabsorption was materially advanced by the work of Sirota[23] who studied Tm PO_4 in three patients before and after removal of parathyroid aden oma Data from one of these cases is presented in Figure 3 As

can be seen, the patient who was excreting large amounts of phosphate preoperatively (with a low Tm PO_4) showed a marked increase in Tm PO_4 after operation with a cessation of phospha turia One may visualize such patients as receiving a continuous endogenous infusion of parathyroid hormone in large amounts

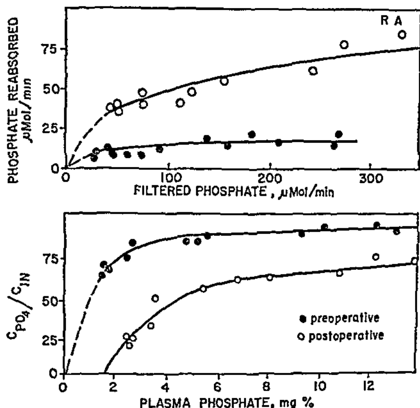


Figure 3 Observations on phosphate Tm in a hypoparathyroid male showing the effects of parathyroid extract From Hiatt and Thompson (18)

from their own over producing tumors Futhermore *it is more* likely that this is an overdose of normal parathyroid secretion than would be the case with the injection of exogenous para thyroid extract

Similar results have been presented by Hiatt and Thompson[18] from studies of a man with postoperative hypoparathyroidism In

this person Tm PO_4 was determined before replacement therapy, and, as can be seen in Figure 4, his Tm was around 170 μMol/minute Two increasing levels of parathyroid therapy then resulted in a marked reduction in Tm PO_4 This went down to about 140 μMol/minute at 100 USP units daily for 5 days and 70 μMol/minute when the extract dose was increased to 900 units a day for 3 days These results represent a neat reverse comp-

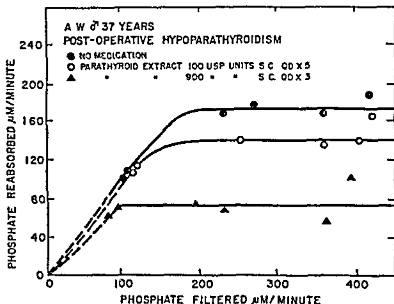


Figure 4 Observations on phosphate Tm in man before and following removal of parathyroid adenoma. Note the increased capacity to reabsorb inorganic phosphate postoperatively From Sirota (Unpublished)

liment of Sirota s shown in Figure 3 It is important to record that in neither Sirota s nor Hiatt and Thompson s subjects were there accompanying changes in renal blood flow or filtration rate

These observations on man are to the author s knowledge the first demonstrations of a definite parathyroid effect on phosphate transport without concomitant alterations in renal blood flow or filtration rate Furthermore, they suggest the idea that the effect

might depend upon a conditioning or adaptation of the transport mechanism that is to say that parathyroid hyperactivity extending over some time is a requisite for demonstrating the parathyroid effect on the capacity of the kidney to transport phosphate

Another example of such an adaptive aspect of the parathyroid Tm PO_4 interrelation is seen in the studies of Foulks[24] who showed that an animal kept for some time on a high phosphate diet will then respond with a lowered Tm PO_4 to a single injection of parathyroid extract Presumably the high phosphate diet stimulates the parathyroid and in some way adapts the renal tubules so that the single challenging injection of parathyroid will lower Tm PO_4

The parathyroid secretion has recently been shown to affect phosphate transport in a quite different and interesting way in the chicken Levinsky and Davidson[25] have shown that there is an inconstant secretion of phosphate by the chicken kidney tubules both at endogenous and elevated plasma phosphate levels However the injection of parathyroid extract into the leg vein and hence the renal portal system is followed in all cases by an increased rate of phosphate secretion in the kidney on the injected side Again this change was noted to occur without any alteration of glomerular filtration This direct effect of parathyroid extract on phosphate secretion in the chicken is illustrated in Figure 5

One wonders after seeing these data on the chicken whether there is a bidirectional flux of phosphate in the renal tubule An acceleration of the secretory limb of such a system would produce a fall in the net reabsorption that is observed in man and dog following parathyroid administration Although the recent observations of Pitts *et al*[26] using the stop flow technique of Malvin and Wilde[27] show only one overall site of phosphate reabsorption in the proximal tubule of dog the experiments do not rule out the possibility of a bidirectional phosphate flux in this one nephron segment

It is now interesting to note in relation to our hypothesis associating phosphate transport and phosphate metabolism that a recent study points to a possible relationship of this sort in the case of the parathyroid effect on phosphate transport in the

kidney De Verdier[23] injected rats intraperitoneally with P^{32} thirty minutes following the administration of saline or para thyroid extract (0 2 USP units per gram body weight) The kid neys were then removed, freed of protein with trichloracetic acid,

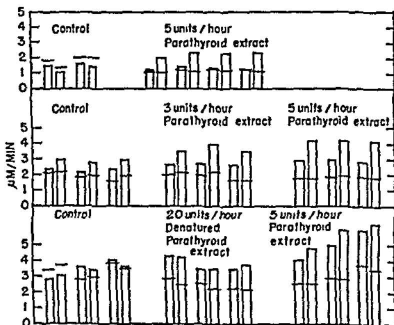


Figure 5 Experiments on the chicken showing the accelerated rate of phosphate excretion by the renal tubule following administration of parathyroid extract Each pair of bars represents right and left kidneys. Open bar represents the infused side stippled bar non infused side Horizontal line with each bar represents rate of glomerular filtration of phosphate Total height of bar represents rate of excretion of phosphate Thus bar below the hori zontal line means filtration with net reabsorption while bar above the hori zontal line means filtration with net tubular excretion of phosphate From Davidson and Levinsky (25).

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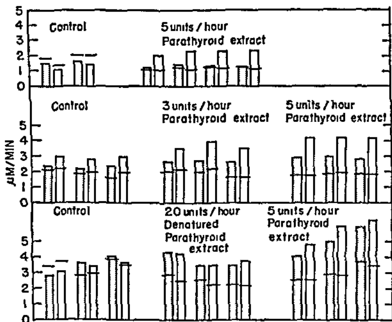


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injected than from the saline control rats From the fact that this difference was most marked in the uridine diphosphate fraction, De Verdier concluded it was most likely that the turnover of phosphate in uridine diphosphate or a derivative of it was stimulated by parathyroid extract This specific conclusion seems a bit too derivative from the evidence presented in the paper, but nevertheless a general stimulation of phosphorous turnover in kidney after parathyroid extract is a most provocative observation It should be repeated and extended with precursor product studies of Zilversmit[7] in animals treated chronically with parathyroid and those on low and high phosphate diets with single and repeated parathyroid hormone injections

The increased P^{32} turnover in the organic phosphorous of the kidney after a single parathyroid injection may be merely the first manifestation or first phase of an alteration in phosphate metabolism which only later, when repeatedly stimulated, manifests itself as a more fully altered metabolic change with an associated reduction in phosphate transport capacity The evidence indicating a need for continued stimulation in dog and man, with either high phosphate diet or repeated parathyroid injections suggests the possibility of a biologic 'adaptation' of the phosphate carrier system in these species similar perhaps to those adaptations seen in specific enzyme systems like tryptophane peroxidase of liver[29] β galactosidase of bacteria,[30] and renal glutaminase in acidosis[31,32] The first are substrate induced adaptations The adaptation in renal glutaminase, although not strictly analogous to the β galactosidase in bacteria, may represent an effect of acidosis *per se* or a substrate induction secondarily associated with the acidosis The parathyroid effect on phosphate transport might be fruitfully studied in terms of a hormone adaptation in one or more of the enzyme systems concerned with organic phosphorous metabolism in the tubular cell membrane

The immediate effect of parathyroid extract on phosphate secretion in the chicken kidney would argue against such a prolonged induction theory in this species However, it is not difficult to imagine that the hypothetical site affected by parathyroid might require induction' in one species and not in another or that the requirements for induction in man or dog might vary from indi

vidual to individual and in any one individual with changing diet and/or state of endogenous parathyroid activity In the absence of organic phosphorous turnover studies in the chicken kidney it is difficult to correlate the parathyroid effect on phosphate secretion in that animal with any intermediary metabolic phe nomena involving phosphate It is hoped that such metabolic studies on the chicken kidney will also be done

B *Effect of glucose and phlorizin*—It has been shown[2,33] that the infusion of glucose into dogs that are receiving in *organic phosphate in amounts sufficient to saturate the renal trans* port capacity will cause a marked drop in Tm PO_4 Conversely the administration of phlorizin in doses sufficient to reduce Tm glucose will cause a striking increase in the tubular capacity to reabsorb phosphate These effects are seen in the experiment of Table I performed on the dog

The studies of Dratz and Handler[34] are of interest in the light of these observations since these authors have studied some aspects of renal phosphorous metabolism during both glucose in fusion and phlorizin injection In their experiments performed on cats one group of animals was infused intravenously with glucose until frank glycosuria was established P^{3} was then given intraperitoneally and 10 to 60 minutes later the kidneys were removed and total acid soluble phosphorous fractionated A sec ond group of cats received phlorizin (50 mg /Kg intravenously) Thirty minutes later when the phlorizin induced glycosuria was present P^{32} was likewise given and 10 to 60 minutes later kidneys were removed and phosphorous fractions isolated Total phos phorous concentration and relative specific activity of P^{32} in each of several phosphorous fractions were then determined The re sults of experiments with the glucose loaded cats are graphically summarized in Figure 6

In these animals the relative specific activity of P^{32} remained unchanged in glucose 1 P and glucose 6 P fractions however it was elevated in the ATP ADP fraction The total amount of in organic phosphate was decreased in the glucose loaded kidneys A decrease in the total concentration of inorganic phosphate in the kidney of the dog is what one might expect during glucose load

TABLE I

EFFECTS OF GLUCOSE AND PHLORIZIN ON THE MAXIMAL RATE OF INORGANIC PHOSPHATE REABSORPTION[33]

Total Concurrent Time min	Urine Flow ml/min	GFR ml/min	Inorganic Phosphate			Glucose		
			Filtered μMol/min	Excreted μMol/min	Reabsorbed μMol/min	Filtered mg/min	Excreted mg/min	Reabsorbed mg/min
	Dog Ca				Experiment 4			
0	Infuse PO_4 Buffer pH 7 4, 0 1 mMol/ml Creatinine 0 6% 6 ml/min							
10	Creatinine Prime 2 0 Gm in 20 ml water intravenously							
32	Infuse PO_4 Buffer pH 7 4, 0 08 mMol/ml Creatinine 0 6%, 6 ml/min							
52– 62	5 2	100	386	252	134	160	2 0	158
62– 72	7 9	101	410	307	103	169	2 0	167
72– 79	Glucose Prime—25 Gm in 100 ml water intravenously							
73	Infuse PO_4 Buffer pH 2 4 0 08 mMol/ml, Glucose 1 0 mMol/ml, Creatinine 0 6%, 6 ml/min							
100–110	16 3	101	385	324	61	870	411	459
110–120	13 0	110	422	328	94	960	386	574
120–130	Phlorizin 500 mg in 100 ml $NaHCO_3$ 25 mMol/L intravenously							
135–145	10 2	91 2	440	271	169	684	643	41
145–155	8 0	96 4	458	298	160	644	581	63

ing because, as we have seen, glucose loading causes a lowered rate of phosphate reabsorption in that species [33] In view of the cat observations of Eggleton and Schuster,[4] however, one cannot say whether such an expected situation would in fact be seen Eggleton and Schuster found that glucose appeared to stimulate phosphate transport rather than depress it as we have seen to be the

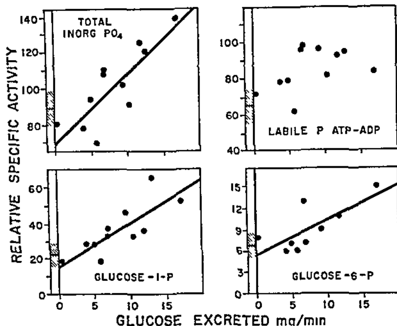


Figure 6 Relation between glucosuria and the specific activities of phosphorous fractions isolated from kidneys of phlorizinized cats 20 minutes after injection of P 32 Shaded areas indicate the mean and standard deviation of the specific activities observed in control cats From Dratz and Handler (34)

case in the dog These authors point out however, that this glucose stimulation in the cat might be the result of the depressed *glucose reabsorption seen in this species at high glucose* loads If this were the case the effect would be more analogous to the situation in the dog where glucose transport is depressed by

phlorizin under these circumstances phosphate transport is ac celerated No data on the effect of phlorizin on phosphate reabsorption are reported in the cat studies Needless to say such in formation would be of great value

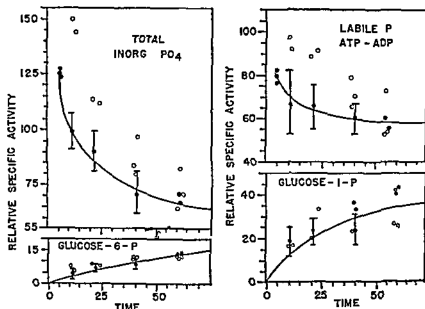


Figure 7 Relative specific activity values of phosphorous fractions isolated from kidneys of glucose treated (o) and control (•) cats Mean and Stand ard deviation are shown where four or more observations were obtained. From Dratz and Handler (34)

Although Dratz and Handler do not report specific figures for the total amount of ATP ADP in the two kidney groups the increased relative specific activity of P^{32} in this fraction they say may correlate with this reduced total inorganic phosphate con tent of these glucose loaded kidneys Thus in view of the definite interrelations between glucose and phosphate transport in dog and cat kidney it would seem fruitful to view these changes in phos phate content and metabolism in this light and repeat the P^{32} studies in the dog where the interrelations are a bit more clear cut

In the kidneys from phlorizined cats a different picture was seen in the various phosphorous fractions but again a picture that is consistent with the changes in phosphate and glucose trans port seen after phlorizin Such an explanation presupposes in the cat a phlorizin effect like that in dog and, as was mentioned above, cat data are not available Thus the following discussion must bear this reservation in mind The results of Dratz and Handler s phlorizin experiments are shown in Figure 7 There was found to be an increased relative specific activity (R S A) of P^{32} in both glucose phosphate esters (glucose 1 P and glucose 6 P) and the ATP ADP system However, the increase in P^{32} R S A was statistically significant only in the ATP ADP fraction at 20 minutes after phlorizin In all these fractions the P^{32} R S.A in creased with the extent of the glycosuria and thus one concludes, with the extent of the phlorizin action

The apparent increase in ATP ADP activity after phlorizin seen in these cats is of interest in the light of the increased trans port of phosphate seen after phlorizin (Table I) Although Dratz and Handler were discussing their results mainly in the light of glucose transport, they really seem more *interesting in relation* to the transport of phosphate For instance one wonders whether there is a relation between the phlorizin induced elevation in phosphate reabsorption and the increased R S A of phosphate in the ATP ADP seen in the phlorizinized kidney or whether ATP-ADP are involved in the carrier mechanisms for both glucose and phosphate These studies with glucose loading and phlorizin glycosuria seem to point in that direction and emphasize the tre mendous need for more work in this most promising and im portant area

C *Effect of 2,4-dinitrophenol on Tm PO_4 and phosphate metabolism*—It is well known that the coupling of phosphoryla tion with biological oxidation in tissues is markedly disrupted by 2 4 dinitrophenol (DNP)[35] The work of Green *et al*[36] and Hunter[35] indicates that DNP acts under certain conditions in mitochondria as an ATPase accelerator Thus *during oxidation in* the electron carrier chain the phosphorylation of ADP to ATP is normal, but the ATP is hydrolized immediately to ADP with the

result that there is a reduced or completely absent *net* uptake of phosphate Respiration, however, continues at a normal or even accelerated pace in the presence of 2,4 DNP since supply of ADP is by no means a rate limiting factor

For these reasons it was of interest to test the effect of DNP on the capacity of the tubules to transport phosphate because a positive *in vivo* effect of this agent would be an important additional link in the chain of evidence that seems to support the hypothesis that relates phosphorous metabolism with phosphate transport in a variety of cells

In Figure 8 are presented experiments on two dogs showing that DNP in a dose of 10 mg /Kg causes a marked reduction in TM PO_4 * This effect which appears within twenty minutes after administration of the inhibitor seems to be transient in relation to the total duration of the DNP effect on the whole animal In Experiment 1, and to a lesser extent in Experiment 2, Tm PO_4 was returning toward control values by 60 minutes after the drug was given But the dog continued to hyperventilate and be hyperthermic for an additional eight hours or so thereafter DNP in these doses has not been shown to lower glomerular filtration or renal blood flow sufficiently to account for this fall in phosphate transport

The possibility must also be borne in mind that the extreme hyperventilation associated with the DNP injection might be related to the depression of phosphate transport Malvin and Lotspeich[37] have shown that dogs artificially hyperventilated in room air at 11 liters per minute show a markedly reduced Tm PO_4 Under these circumstances the respiratory alkalosis is accompanied by a profuse bicarbonaturia The transient nature of the DNP depression of Tm PO_4 might argue against this explanation however, because the hyperventilation in the DNP treated dogs continued long after the Tm PO_4 effect had passed and in

*Mudge has stated without presenting data (*Am J Physiol* 161 175 1950) that DNP does not affect the maximal rate of phosphate reabsorption in dog The difference between this statement and the experiments presented here is unexplained The transient nature of the DNP effect in some animals means that it could have been missed Some such factor may explain the discrepancy

the experiments of Malvin and Lotspeich Tm PO_4 was still de pressed fifty minutes after the beginning of the hyperventilation

These experiments, therefore, must be interpreted with cau tion in terms of any relation between phosphate transport and the specific phenomenon of oxidative phosphorylation The DNP may be affecting the phosphate carrier quite independently of any

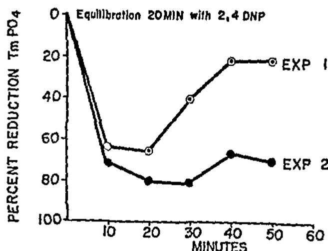


Figure 8 Two experiments showing depression of phosphate Tm in the dog following injection of 2 4-dinitrophenol in a dose of 10 mg/kg intra venously (Unpublished observations from the author's laboratory)

effect on the oxidative phosphorylation system of the cell, as is the case with the DNP inhibition of phosphate transport in yeast and bacteria There the membrane reactions in phosphate transport are specifically and separately blocked The experiments viewed thus are presented as interesting observations in which it seems possible that DNP may likewise be affecting the membrane carrier for phosphate in the kidney Whether this carrier also involves adenyl polyphosphates in the tubular cell membrane is an open and unproven hypothesis at present But in the light of the experi ments discussed here it seems a worthwhile question for experi mental study

Summary

We have examined a great deal of evidence from a variety of biological species and have seen a number of instances where the transcellular movement of orthophosphate seems to involve a membrane carrier that forms an organic phosphate complex In some cases, as in the erythrocyte, this carrier appears to involve the ATP ADP system Subsequent work may alter the exact identity of the membrane carrier and may add new elements to the complete mechanism What has been attempted here is the erection of an hypothesis derived from an examination of the available comparative evidence which suggests that phosphate transport in cells of higher forms may involve the formation of similar organic phosphate complexes It is hoped that this treatment of the subject, without stating a dogmatic conclusion about the nature of phosphate transport in the kidney, will stimulate further research in this area That such research may alter or completely upset the thesis developed here is a real possibility

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III

AMINO ACIDS AND GLUCOSE

It now seems logical in the development of our thesis to focus attention on two of the major components of cellular chemistry, namely, amino acids and glucose The amino acids are at the center of protein synthesis and growth, and, therefore, the trans port of these substances in and out of cells all over the body must be related to the changing requirements of this process Glucose, in like manner, is probably the main energy source for cell life, at least in some tissues such as brain and muscle

The kidney has an active glucose metabolism, it carries out glycogen synthesis from glucose and non-glucose sources, and in certain conditions, such as von Gierke's disease and uncontrolled diabetes, glycogen accumulates in its parenchyma Besides its role in energy metabolism, glucose transport deserves special at tention in this treatise because the mechanism has, like amino acid transport, been exhaustively studied in a variety of cell types and intact organs

Some excellent information is available about the transport and utilization of other metabolites such as lactate, ketones, α ketoglutarate, and pyruvate Some of this information will form the subject matter of the next chapter where attention will be focused on the tricarboxylic acid cycle However, because a more limited treatment seemed to afford a greater unity in the present essay, it will be confined to a consideration of the amino acids and glucose As we will see, certain groupings of dysfunction in hereditary disease make it natural to consider the amino acids and glucose together and immediately following our discussion of phosphate In this, as in the next chapter, one sees a fruitful area for studying the transport of metabolites and its relation to their function in the cellular metabolism

Amino Acids

Amino acids circulating in the blood are in a dynamic equilibrium with the amino acid pool inside cells, and this pool in turn is in a continual state of turnover as a result of the anabolic catabolic cycle of proteins For this reason the movement of amino acids in and out of cells is an integral part of the overall process of protein homeostasis It is this amino acid transport process that we will examine, first in the kidney, to a lesser extent in the intestine, and then in several other cell types where the process has been studied with significant results These data will then be related in so far as they can, to the dynamic state of body proteins in general

Renal transport of amino acids—The first careful studies on amino acid excretion were those of Kirk[1] in 1936 who showed that the total amino acid clearance was very low at endogenous plasma levels but rose markedly when glycine was infused From these studies it was obvious that amino acids are reabsorbed by the tubules Doty[2] subsequently showed that histidine and tyrosine at moderately elevated filtered loads are almost completely reabsorbed while the N methyl and N acetyl tyrosines are poorly reabsorbed even at low plasma levels In a subsequent study with glycine infusion in dogs, Pitts[3] showed that over a wide range of filtered loads the rate of amino nitrogen reabsorption rises and asymptotically reaches a maximal value at loads some fifteen times those at endogenous plasma amino nitrogen levels

Subsequent studies of this type,[4 5] measuring total amino nitrogen during infusion of a single amino acid, have shown that the individual amino acids are filtered through the glomeruli in proportion to their plasma level, then undergo tubular reabsorption at different rates Such data for five representative amino acids are shown in Figure 9 Glycine reabsorption is high and still rising over the range of filtered loads shown here With only slight elevation of plasma levels of L arginine and L lysine, a Tm for these amino acids is reached, they are reabsorbed relatively 'poorly' L-glutamic acid and L-leucine fall in between glycine and the L forms of arginine and lysine DL alanine, DL aspartic

amino acids, the authors with good reason classed histidine among the neutral rather than the basic group

The acidic amino acids, glutamic and aspartic, are of especial interest from several standpoints They are the least effective inhibitors of transport of either the neutral or basic group In addition, the excretion of these acidic amino acids is not affected in any general way by the other groups but rather by individual amino acids in different ways For instance, the excretion of both aspartic and glutamic acids is most profoundly enhanced by alan ine, and alanine is the only amino acid affecting transport of *both* aspartic and glutamic acids *Singly,* histidine and asparagine greatly enhance glutamic acid excretion, and glutamic acid and glutamine in turn similarly increase the excretion of aspartic acid

It is significant, as the authors point out, 'that the amino acids mentioned—alanine, glutamine, asparagine, glutamic acid, aspartic acid and histidine—either are, or may be readily con verted to, the three amino acids participating in transamination reactions It is also of great interest in this regard that alanine is the only amino acid inhibiting the transport of both glutamic and aspartic acids Alanine, glutamic acid and aspartic acid con stitute the main element of the two transamination mechanisms which have thus far been isolated These are (1) The *aspartic glutamic transaminase* and (2) the *alanine glutamic transaminase* The idea that transamination may play a role in amino acid trans port has recently received strong support from the observation by Neame and Wiseman[10] that a portion of glutamic and aspartic acids is transaminated during their transport across the small intestine

Kamin and Handler[9] came to the conclusion that there may be more than one amino acid transport mechanism in the kidney or that one mechanism may transport many amino acids that have markedly different affinities for it As we shall see later in this discussion, there is much evidence to support the role of pyridoxal (vitamin B_6) in the transport of amino acids across the cell membrane in Ehrlich ascites tumor cells Pyridoxal phos phate is also a part of the transaminase enzyme systems Thus some aspects at least of amino acid transamination may participate

in the renal, as well as intestinal, transport of some or all of these metabolites So far as the writer knows, there has appeared no careful study correlating rate of transamination of a number of amino acids by kidney or intestine with their rate of transport in these organs

The studies of Kamin and Handler were carried out with specific microbiological assay methods for each amino acid Their observation that the infusion of a single amino acid causes the appearance of other amino acids in the urine means that the earlier experiments of Pitts[4] and Lotspeich and Pitts[5] may have contained an experimental error In those experiments plasma and urine total α amino nitrogen was measured during infusion of a single amino acid Thus, in the light of Kamin and Handler s findings the urinary α amino nitrogen constituted not only that from the single amino acid being administered but, in addition, that from other amino acids whose tubular reabsorption had been inhibited Although perhaps not a large error, this phenomenon would have caused an apparent lower rate of reabsorption of an individual amino acid (in terms of α amino nitrogen) in direct proportion to the extent of its inhibition of the transport of other endogenous amino acids from the glomerular filtrate

Kamin and Handler[11] in a separate study have also observed absorption of amino acids in the small intestine They employed the Cori isolated loop technique in the rat In these studies a second amino acid almost invariably inhibited the absorption of the first and no obvious pattern of competitive interrelations between amino acids emerged as it did in the kidney In a more recent study, however Wiseman[12] has found such a pattern for intestinal amino acid absorption Using the isolated sac of everted golden hamster small intestine he has studied the preferential transfer of individual amino acids from mixtures of these acids in the intestine He found that certain L amino acids are actively transported against concentration gradients (The D isomers are not so transported, as Matthews and Smyth have shown [13]) Those acids actively transferred are the L forms of proline histidine and methionine Again as in the kidney, the basic acids, lysine and ornithine, are not actively transported Those amino acids with active transfer mechanisms compete with one another when

Christensen s experiments glutamic acid was an exception to the general competitive pattern Instead of blocking the transfer of other amino acids it actually caused a higher concentration of other amino acids in cells Presumably through stimulating trans amination other amino acids were formed This is probably why glutamic acid is not essential from a dietary standpoint it can be formed from other amino acids and in turn lead to the produc tion of others in its central position in the transaminating system

From these studies it is easy to see why Cannon s rats had to have their essential amino acids all at once and in the right proportions Presumably the specificity of the amino acid carriers at the cell membrane will allow transfer of the whole mixture of amino acids in their proper proportions if they are presented in the right constellation But if the mixture is imbalanced they are not carried into cells in the right proportions for protein syn thesis and no growth occurs Viewed in this light competitive interrelations among the several amino acids for transport across cell membranes may have some teleological meaning

For this reason it is perhaps an artifact unrelated to the real function of amino acids when one observes the maximal reab- *sorptive transfer of one amino acid in the absence of simultaneous* loading with the whole constellation of amino acids If such experiments are to have real meaning in terms of dynamic pro- tein metabolism they might better involve a study of the simul taneous reabsorptive rates of the whole group of amino acids at their own endogenous plasma levels

With this discussion of amino acid transport in kidney in testine and certain tissue cells and a consideration of the relation of this process to the general problem of protein metabolism we are now ready to examine in some detail a body of recent work describing the chemical nature of the amino acid carrier itself as it has been studied in certain special cells

The amino acid carrier mechanism—The transfer of amino acids into a variety of cell types has been the subject of a number of brilliant studies Spiegelman and Halvorson[16] have observed this process in yeast and have related it to adaptive enzyme forma tion in that species Gale[17] has studied amino acid uptake in

staphylococci both in the intact and sonically disrupted cell and has shown that only under certain special conditions is the transfer of the amino acids associated with the synthesis of new protein For instance *Staphylococcus aureus* can take up C^{14} glutamic acid which will then accumlate in the free form in the cell and appear in its protein without any new protein having been formed But for this uptake to be accompanied with new protein synthesis all the essential amino acids must be present along with an energy source and the proper nucleic acids

Thus in his studies Gale has clearly differentiated the transfer process by which the free amino acid is concentrated within the cell (without concomitant protein synthesis) from the process whereby amino acid transfer is coupled with synthesis of new protein This distinction between uptake and protein synthesis so clearly seen in the microorganism in the presence of one single amino acid again suggests the unrelatedness of the transport of a single amino acid at maximal rates in the kidney to the process of protein metabolism

In another series of studies Christensen and his col leagues[18 19 20 21] have analyzed the concentrative transfer of amino acids in rat diaphragm erythrocytes reticulocytes and most extensively in the mouse Ehrlich ascites tumor cell They are free growing in the ascitic fluid of the innoculated mouse and can be easily harvested by aspiration of the fluid from the peritoneal cavity

Using these cells Christensen presents convincing evidence that the active transport of an amino acid across the cell mem brane into its free amino acid pool involves chemically discrete reactions leading to the formation of a stabilized Schiff s base be tween pyridoxal (vitamin B_6) and a metal chelate of the amino acid These reactions as visualized by Christensen are shown in Figure 10

Having thus stated the conclusion first let us back track a little and examine in some detail the overall characteristics of amino acid uptake in these cells First we must examine the evi dence supporting the statement that the transfer process is active and involves a carrier that reacts chemically with the amino acid Then we will examine briefly the evidence that these reac

tions involve formation and dissociation of a Schiff s base like that in Figure 10 And finally we will ask about the relation of this work to the problem of amino acid transport in the renal tubule and elsewhere

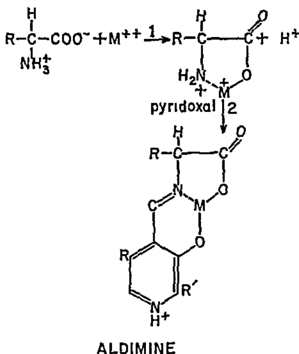


Figure 10 Reactions involved in the formation of a stabilized Schiff's base between metal chelate of an amino acid and pyridoxal postulated to take place in the transport of amino acids across certain cell membranes From Christensen Riggs Aspen and Mothon (19)

The uptake of amino acids[18] by the ascites tumor cells dia phragm or nucleated erythrocytes *in vitro* results in a higher con centration of the free amino acid inside the cell than in the extra cellular medium For this reason the process is termed active It requires energy and has been termed concentrative transfer by Christensen The curve relating uptake to amino acid con centration in the medium has the characteristics of an adsorp- tion isotherm thus indicating that there is a carrier of limited

capacity in the membrane These characteristics for the uptake of tryptophane are seen in Figure 11 The process is temperature dependent beyond the limits of a temperature facilitated diffusion It is in addition inhibited by lack of oxygen cyanide and other inhibitors of biological oxidation

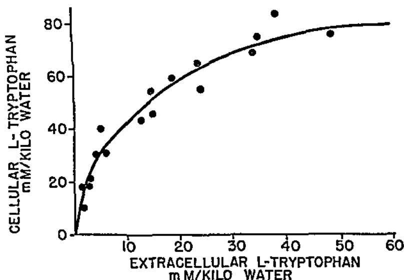


Figure 11 Relation between extracellular and intracellular tryptophane in mouse Ehrlich ascites tumor cells Temperature 37° C time 2 to 4 hours From Riggs Coyne and Christensen (43)

These facts indicate that chemical reactions are involved in the carrier mechanism That the carrier is not permanently bound by the amino acid is shown in the ingenious studies of Heinz[22] who observed that the transfer rate of 1 C^{14}-glycine into ascites tumor cells was the same both before and after the cells had been loaded with unlabelled glycine Thus the carrier after re acting with the amino acid and dissociating from it is free to com bine with additional amino acid and then release it at a point of higher concentration within the cell

The first clue to the nature of the chemical reactions involved in this mechanism came from the observation that *pyridoxal* stim

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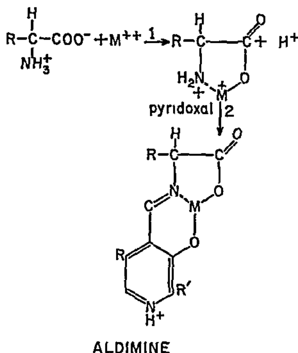


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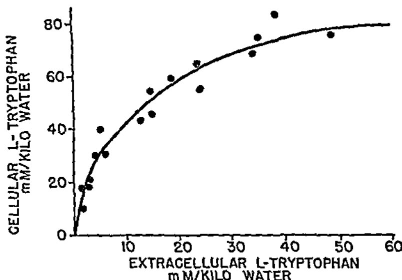


Figure 11 Relation between extracellular and intracellular tryptophane in mouse Ehrlich ascites tumor cells. Temperature 37° C time 2 to 4 hours From Riggs Coyne and Christensen (45).

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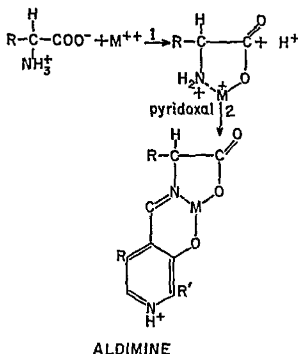


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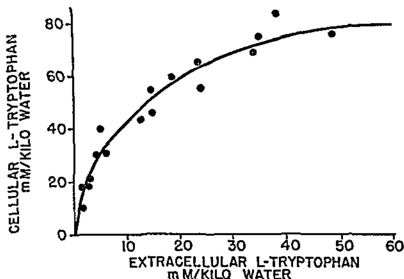


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These facts indicate that chemical reactions are involved in the carrier mechanism That the carrier is not permanently bound by the amino acid is shown in the ingenious studies of Heinz[72] who observed that the transfer rate of 1 C^{14}-glycine into ascites tumor cells was the same both before and after the cells had been loaded with unlabelled glycine Thus the carrier after re acting with the amino acid and dissociating from it is free to com bine with additional amino acid and then release it at a point of higher concentration within the cell

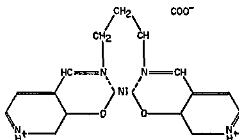
The first clue to the nature of the chemical reactions involved in this mechanism came from the observation that *pyridoxal* stim

ulates the concentrative transfer of certain amino acids in the ascites tumor cells and conversely that the process is less rapid in cells from pyridoxal deficient mice[23,24] Metzler, Ikawa, and Snell[25] had shown in an extensive series of studies that metal *chelation and Schiff's base formation are involved in the cat*alytic function of pyridoxal in several reactions involving amino acids Among these are transamination, decarboxylation, and the serine threonine interconversion These workers also showed that 4 nitrosalicylaldehyde, the benzene analogue of pyridoxal, can also form stable Schiff s bases with metal chelates of some amino acids[26] Therefore, the subsequent observation that 4 nitrosalicyl aldehyde, like pyridoxal, also stimulates the cellular uptake of certain amino acids[18] added strongly to the belief that similar metal chelation and Schiff s base formation with pyridoxal is involved and perhaps rate limiting in the carrier reaction in amino acid transfer

The 4 nitrosalicylaldehyde stimulation of amino acid transfer immediately furnished a key aspect of the nature of the pyridoxal function in the transfer process Since it is unlikely that 4 nitrosalicylaldehyde can be altered enzymatically by phosphorylation or that it can affect the energy yielding reactions of the cell, *its stimulatory effect on amino acid transport indicates that* pyridoxal, whose role it mimics, does not necessarily function in the carrier reactions in a manner analogous to the function of coenzymes in tissue respiration

These observations led Christensen and his colleagues to a definitive study[20] in which they showed that a number of circumstances conditioning the formation of stabilized Schiff s bases be*tween pyridoxal and amino acid metal chelates also condition,* in a parallel way, the concentrative transfer of amino acids in the ascites tumor cell Among these parallel circumstances are the following (1) α or β amino acids are transported but not γ This function correlates with the capacity of these amino acids to chelate with copper (2) Critical distance between amino and carboxyl groups of an amino acid determines both transferability and Schiff s base *formation* (3) *Substitution of the* 3 hydroxyl group of pyridoxal with a methyl group blocks the capacity of pyridoxal both to stimulate amino acid uptake and to form Schiff s

base with the chelated amino acid (4) Studies with α γ-diamino butyric acid[71] showed that this non metabolized amino acid is so intensively concentrated by the ascites tumor cells that they swell up and are ultimately destroyed This diamino amino acid avidly forms a metal stabilized Schiff s base with pyridoxal in the ratio of one amino acid one metal two pyridoxal The structure of this compound with nickel as the metal can be represented as follows



If one prevents the formation of this pyridoxylidene derivative of the diamino acid by substituting two N methyl groups at the α position the concentrative uptake of the acid by the cells is abolished

This, and additional evidence, has led Christensen and his group to conclude that amino acid transport in the ascites tumor cell involves formation of a metal stabilized Schiff s base between amino acid and pyridoxal along the lines shown in Figure 10 In the transport scheme shown in Figure 12 this amino acid complex is formed in the membrane and the reaction is probably enzy matically catalyzed Energy is not required for its formation or its dissociation when amino acid concentration inside and outside the cell are equal However, energy is required if the carrier is to *concentrate* amino acids inside the cell Although there are a number of ways this energy could be introduced, one way, as Christensen has suggested would be to present the carrier in a higher state of chemical potential or energy form, at the outer side than the inside of the cell membrane

Whether these studies bear any relation to the amino acid transport mechanism across the renal tubule or intestine is hard

to say at the moment The kidney contains pyridoxal enzymes such as *transaminase* But no studies have appeared analyzing amino acid transport in the kidney in terms of the carrier system described above for the ascites tumor cell

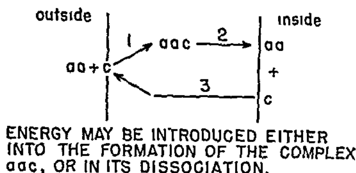


Figure 12 A conception of active transport. Discussion in text From Christensen Riggs Aspen and Mothon (19)

Fridhandler and Quastel[27] have attempted such a study in the isolated hamster intestine but without much success They found that deoxypridoxine, a molecular analogue of pyridoxine in hibits amino acid transport across the intestine But the effect of this agent is not limited to amino acids for glucose transport is blocked as well at the high levels of deoxypyridoxine used This observation with deoxypyridoxine is by no means decisive because the experiments in which it was used were done at levels of the aromatic aldehydes which are inhibitory to amino acid transport even if pyridoxal itself is used At these high levels of pyridoxal there occur marked shifts in electrolytes between cells and sur rounding medium, which cause the cells to swell Under these circumstances amino acid transport is inhibited Thus these ex periments on the intestinal absorption of amino acids are by no means a critical test of the Schiff s base-chelation theory

It would perhaps be a more hopeful approach to study the tubular transport of substituted amino acids which are known not to form Schiff s bases of the kind discussed One recalls in this regard Doty s[2] observations that while tyrosine is almost com

pletely reabsorbed in the dog kidney, N methyl L tyrosine and N acetyl L tyrosine are not These N substituted derivatives would not form the Schiff s bases with tyrosine Again the use of some of Christensen s amino acids lacking α hydrogen[28] or amino acids in which carbon chain length and number of amino groups are varied would provide an interesting basis for examining the relevance of the Schiff's base idea for renal amino acid transport The fact that arginine and lysine are poorly reabsorbed by the kidney is a relevant observation here Both these amino acids do not form strong chelates because their nitrogen atoms are too far apart in the carbon chain

The work of Christensen s group is presented and discussed here in some detail even though its relevance to the problem of renal amino acid transport is an open question at the moment It represents one of the most carefully worked out studies of a carrier mechanism in cells of higher forms and it points the way toward the type of analysis that is required before analogous renal mechanisms can be clearly understood

Glucose

The relation between glucose transport and its metabolism has been of interest to physiologists for many years Although this relation is by no means completely understood at the present some recent studies have clearly shown that the two processes have features both separate and in common

That glucose as well as a number of other sugars is trans ported by a variety of cells is well known In particular we shall emphasize that the process has many similar features in both kid ney and intestine For instance, in both organs glucose transport is active, that is, can be carried out against chemical gradients as described for phosphate and amino acids In both kidney and intestine the mechanism is inhibited by phlorizin and exhibits maximal transport capacity This phenomenon referred to as Tm glucose, is shown strikingly in the data of Figure 13 taken from the classical experiments of Shannon and Fisher[29] Here it is seen that glucose reabsorption parallels plasma glucose (and

hence glucose filtration) until a maximal rate is reached Beyond this point the transport rate remains *constant despite further* increases in the quantity of glucose presented to the tubules Shannon and Fisher's *analysis of this* mechanism was discussed earlier (Chapter II) in relation to phosphate where similar characteristics are seen

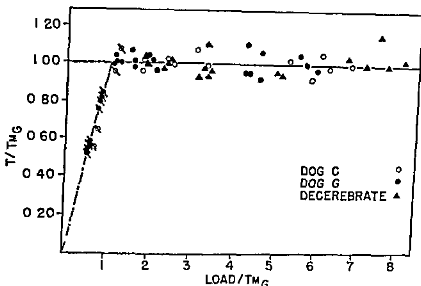


Figure 13 Relationship between glucose reabsorption and its filtered load in the dog *Tm G for each dog was taken as the mean of all observations at* plasma concentrations above the level of obvious glycosuria The broken line represents the relationship to be expected if reabsorption remains complete until load/Tm G equals one The crossed symbols represent observations where more than 99 per cent of the filtered glucose has been reabsorbed From Shannon and Fisher (29)

Such an active mechanism undoubtedly has both passive (diffusion) and active components determining its maximal capacity However, in view of the relative impermeability of most biological membranes for glucose and its relative insolubility in lipids the active carrier component of the process probably represents the major rate limiting factor in glucose transport The nature of this carrier in cell membranes must now receive our attention

Vezár[30] developed the hypothesis that sugar transfer in intestine and kidney involves phosphorylation of the sugar at some stage in the cell, and he believed that the capacity to phosphorylate limits the transport capacity. On the basis of this concept Lundsgaard thought that phlorizin inhibited glucose transport by blocking its phosphorylation, a theory discussed later (Chapter VI). Largely on the basis of Lundsgaard's own work, this theory was abandoned. Its rejection was strengthened by the earlier observation of Cori that unnatural synthetic sugars, such as 3-methyl glucose, are also actively transported across the intestine.[31] It has now been conclusively shown by Sols[32] that neither 3-methyl glucose nor galactose, which is also absorbed by the intestine, are phosphorylated by any hexokinase present in the intestinal mucosa.*

In striking contrast to this evidence arguing against a phosphorylation theory of glucose transport in kidney and intestine, the beautiful studies of Rothstein and his colleagues[33] strongly *support* a phosphorylation theory for this process in yeast. Rothstein found that glucose uptake by yeast is exquisitely sensitive to uranium in the form of the bivalent uranyl ion (UO_2^{++}). He studied the distribution of uranium between yeast cells and surrounding medium with varying concentrations of a number of uranium complexing agents. Of all these agents studied, only the multiphosphate compounds gave uranium complexes with a stability of similar magnitude to that of the yeast uranium complex. In addition, formations of the uranium phosphate and the yeast uranium complexes were affected similarly by pH and mass law conditions. On the basis of these studies Rothstein concluded that glucose uptake by yeast involves formation of glucose complexes with polyphosphates in the membrane and that these reactions are similar to, but completely separate from, glucose phos-

*Since this work Diedrich has found that during the intestinal absorption of galactose in the rat, galactose-1-PO_4 is found, indicating that a phosphorylating system for galactose is apparently present. (Diedrich, D. F., Ph.D. Thesis, University of Wisconsin, 1959.)

phorylations that occur during metabolism in the cell interior Using the uranium binding technique, Rothstein has actually measured the number of these polyphosphate glucose carrier sites in the yeast membrane, finding 4 6 × 10^7 sites per cell Although not proven by experiment, Rosenberg[34] has suggested on theoretical grounds that glucose transport across the erythrocyte membrane may likewise involve formation of a metaphosphate ester Thus glucose transport in kidney and intestine is obviously different, at least in certain fundamental respects, from yeast and erythrocytes However, as we will see subsequently, the method of study used by Rothstein and his group would seem to offer real promise when applied with certain modifications to the renal and intestinal situation

Recent studies by Chinard, Taylor, Nolan, and Enns[35] have shown conclusively that the six carbon chain of glucose is not split into triose units during its transport across the renal tubule of the dog Glucose 1 C^{14} was rapidly injected into the renal artery, followed by the collection of serial samples of renal vein blood at close intervals of time thereafter Having established that the radioactivity in renal vein blood was due to the labelled glucose rather than other non volatile compounds, the glucose was degraded microbiologically, and it was found that essentially all the radioactivity was still in the 1 C position This absence of randomization of the labelled carbon during glucose reabsorption in the kidney established the fact that there is no breakdown and resynthesis of the six carbon chain during its transport. Exactly analogous concl[illegible] ached prev

in such an elegant way as Rothstein and his colleagues have done in yeast However Rothstein s method of study could be applied to the problem in these more highly organized cells The use of phlorizin may offer an approach in these organs analogous to Rothstein s use of uranium in yeast Phlorizin in kidney like uranium in yeast appears to bind a membrane glucose carrier and in perfused or *in vitro* systems a study of the kinetics and the chemical nature of this binding should in like manner reveal the nature of the glucose carrier and its concentration on cells in kidney and intestine Until such precise studies are performed we will not understand completely the problem of glucose transport

The phenomenon furthermore is not the same in all mammalian cell types For instance in muscle and lens sugar uptake is facilitated by insulin in contrast to intact kidney intestine or such *in vitro* systems as the Ehrlich ascites tumor cell[37] and slices of kidney cortex[38] In these latter instances a number of sugars penetrate the cells and reach diffusion equilibrium where the sugar concentrations in cell and surrounding medium are equal Here where non active transfer occurs where there is competitive inhibition among the several sugars transferred and where the process is sensitive to phlorizin there is a unique opportunity to study the separate factors of diffusion carrier pores insulin and the role of energy in the overall sugar transport mechanism In those instances where insulin exerts its effect we have still other factors apparently *not operating in either the active transport in* kidney and intestine or the non active kind in the ascites tumor cell or kidney cortex slice Thus sugar transport is really a series of different phenomena in several different cell types Future research will undoubtedly elaborate the common and special features of each different mechanism and explain in more detail the relation between glucose transport and its metabolic assimilation

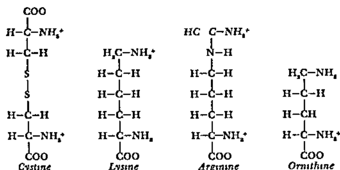
Phosphate, amino acids, glucose—congenital tubular dysfunction—In our discussion thus far we have considered in detail the relation between transport and metabolism in the case

of three major metabolites phosphate, amino acids, and glucose Before concluding discussion of this phase of our topic, it is of interest to recall that these three metabolites show simultaneous dysfunction of their renal tubular transport in at least one clinical condition, Fanconi s syndrome * Here in either the juvenile form or the similar condition seen in adults there is excessive loss in the urine of inorganic phosphate, certain amino acids, and glucose The loss of phosphate leads to chronic hypophosphatemia with demineralization of bone Cystine, making up a large part of the urinary amino acid, being relatively insoluble oftentimes pre cipitates and leads to renal calcinosis In addition, particularly in infants, there is a widespread appearance of cystine crystals in the tissues indicating a more general defect in cystine metabolism This condition has been shown to follow typical Mendelian genetic patterns in a number of families carefully studied The defect seems to be a tubular lesion where the reabsorption of these three substances is specifically absent or nearly so The lesion is in all likelihood a proximal tubular one on both functional and morphologic grounds Phosphate, amino acids, and glucose are probably all reabsorbed in the proximal tubules Darmady and his associates have shown[39] by microdissection techniques that the *proximal tubules in this condition are shorter than normal and* are joined to the glomeruli by a narrow, thin walled swan neck '

Other reabsorptive defects involving amino acids phosphate, *and glucose in greater or lesser degrees of severity* and specificity are known The condition known as *cystinuria* is actually not limited to a defect in reabsorption of cystine One usually sees a proportionally increased excretion of lysine, and if lysine excre tion is high enough arginine also appears in excessive amounts in the urine Occasionally ornithine appears as well

Dent and Rose[40] *have discussed the association of these spe* cific amino acids in this tubular dysfunction syndrome in terms of possible common denominators in their molecular structure

*For an excellent discussion of the clinical tubular dysfunctions the reader is referred to the article by G H Mudge *Am J Med* 24 785 1958



Cystine *Lysine* *Arginine* *Ornithine*

They suggested that they are similar in structure and thus per haps similarly affected in transport In all four there are two posi tively charged amino groups separated by a chain of four to six atoms It has been suggested[41] that their transport could involve the combination of these positively charged amino groups with similarly spaced negatively charged carboxyl groups projecting from alternate glutamate or aspartate residues on a protein surface

In the light of Christensen s work discussed earlier one might visualize an alternative mechanism explaining the association of these amino acids on the basis of a similar difficulty in the forma tion of the amino acid Schiff s base chelate As was pointed out earlier diamino acids such as lysine and arginine do not form strong chelates with metals because their nitrogen atoms are too far apart Such could also be the case for cystine where the two sulfur atoms in the chain move the amino groups even farther apart Whether the nitrogens are too far apart in ornithine to preclude a stable chelate formation the writer does not know

Thus in those amino acidurias where there is fairly normal nephron structure the tubular transport defect, genetically based could be the result of an insufficient or spatially altered com ponent in the carrier mechanism for amino acids which was adequate for the transport of most members of this series of com pounds but inadequate (perhaps in supply of pyridoxal enzyme, or metal) to react strongly enough with those amino acids shown above In view of Christensen s work it would be valuable to as say the kidney tissue of cystinurics for pyridoxine and the trans

aminase enzymes even though the applicability of the Schiff's base-chelation theory to the problem of renal amino acid transport is not yet known The cystinuric in whom there is not the generalized proximal tubular dysfunction of the Fanconi s would seem to be an ideal subject for studying the amino acid carrier This would also be a particularly hopeful approach in a strain of mice with a similar hereditary defect in the renal tubules

Thus in the Fanconi type syndrome phosphate amino acids and glucose (as well as bicarbonate and potassium in some cases) seem to be generally affected in the face of a general proximal tubular lesion with a demonstrated morphological basis However in the case of cystinuria the lesion appears to be more discretely limited to amino acids of a definite structure

In certain cases of renal glycosuria there is also a hereditary and rather specific defect in glucose reabsorption and similarily of phosphate reabsorption in renal hyperphosphaturia It is these specific limited hereditary tubular lesions that offer such fruitful grounds for the study of the biochemical lesion and thus the normal carrier mechanisms for these metabolites Such a study as has been suggested has provided the explanation of another hereditary metabolic defect in the kidney the glycogen storage disease of von Gierke Dr Gerty Cori[42] has shown that there is a marked renal deficiency of glucose 6 phosphatase in the proximal tubule of these patients

Summary

The characteristics of tubular transport of two major components of metabolism amino acids and glucose have been described and examined In both cases these mechanisms have been discussed in relation to the intermediary metabolism of the substance as was done in the case of inorganic phosphate With amino acids and glucose however the immediate relation between their transport and metabolism was not as clear as it appears to be in some cells with phosphate Such a close relation in the case of glucose would be apparent if it could be shown that its transport involves phosphorylation in a way similar to this process in anaerobic glycolysis Although such a scheme seems likely in yeast and some erythrocytes the evidence does not

support it in kidney or intestine In addition the evidence is also against a cleavage of the glucose molecule in its transport in these two organs Although a membrane carrier appears in volved in glucose transport in kidney, its nature remains obscure

The characteristics of the amino acid carrier have been beau tifully worked out in the Ehrlich ascites tumor cell and the scheme appears in some respects to apply to skeletal muscle and liver as well This scheme involves the formation in the mem brane of a stable Schiff s base between an amino acid metal chelate and the vitamin pyridoxal Since pyridoxal phosphate has been shown to act as coenzyme in a variety of reactions in the inter mediary metabolism of amino acids it is tempting to see in this mechanism an adapted form of some of those reactions Thus as Snell and his associates have shown the reactions whereby pyridoxal enzymes act in amino acid transaminations decarboxyla tions and other specific interconversions show characteristics very similar to those involving pyridoxal in the Schiff s base formation during amino acid transport Although this mechanism has not yet been generalized it furnishes a most plausible approach to the study of amino acid transport in a variety of cells At the moment its applicability to the problem of renal tubular amino acid transport although not established seems promising from indirect bits of evidence This comprises similarities in relative rates of transport of different amino acids in the kidney and as cites tumor cells similarities in competitive interrelations in transport in these two situations and similarities in ability to form Schiff s base-chelates and in capacity to be transported in kidney Although by no means wedded to the Christensen theory the writer has discussed it in some detail because it represents the most precise work available on amino acid transport at present Subsequent work may well explain the observed facts on different and equally plausible grounds

We have centered attention briefly on those hereditary situa tions in which the renal tubular transport of phosphate, amino acids and glucose is more or less reduced with a greater or lesser degree of specificity Like mutant *neurospora*, these patients lack the ability to carry out one or more of the specific biochemical reactions in the tubular transport of these metabolites Thus the

identification of the missing reactions will provide the information concerning the steps that function normally It has further been suggested that the increased use of artificially mutant or hereditarily deficient animals may offer a valuable tool in studying the biochemistry of transport in a manner analogous to the study of genetic biochemistry in molds or bacteria

Finally there is a need to study the relation between the transport of metabolites into and out of cells in relation to the assimilative needs of the cells for the metabolite in energy metabolism, synthetic reactions, and growth

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IV

THE TRICARBOXYLIC ACID CYCLE IN KIDNEY

THE SERIES of biological oxidations now known as the Tricarboxylic Acid Cycle were described in their present form by Krebs[1] This sequence of reactions is presented in its conventional form in Figure 14 which will serve as reference for the discussion to follow The cycle is now generally accepted as the final common pathway for the aerobic oxidation of carbohydrate fat and protein in many tissues of a wide variety of living forms

All the organic acids which constitute the intermediate substrates of this cycle have been identified at one time or another in urine of lower animals and man Under a variety of conditions their excretion particularly citric and α ketoglutaric acids shows remarkable variations Although the full teleological reasons for these changes in organic acid excretion are not yet known they appear to play an important role in anion conservation during the renal regulation of acid base balance In addition citrate appears to keep calcium in solution in the urine and thus prevent renal calcinosis

The mechanism of synthesis and excretion of some of these cycle acids in the kidney has presented the observer with a series of facts of great intrinsic biological interest There is now at hand a body of evidence suggesting that some of these organic acids are synthesized by the cycle in the kidney and then secreted into the tubular urine The cycle as it operates in renal tubular parenchyma thus appears to be adapted to function in the synthesis and transport of its own intermediate substrates Furthermore this aspect of the cycle s operation in the kidney appears to constitute the system regulating the excretion of organic acids in

accordance with the changing requirements of acid base regulation and calcium excretion

Of course it also plays an important role in the intrinsic energy metabolism of the kidney Studies are in progress[2] that are attempting to assess the importance of the cycle in the energy

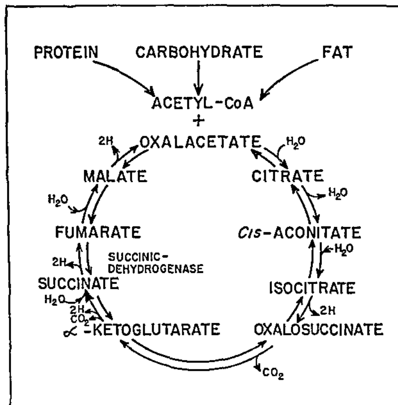


Figure 14 Schematic representation of the tricarboxylic acid cycle

requirements of the kidney These studies have revealed thus far that α ketoglutarate and pyruvate in particular are rapidly extracted from renal arterial blood and utilized by the kidney [2] This process can be shown to be affected by conditions that do not change the transport of α ketoglutarate across the tubule

Thus an interesting field of investigation is opening up in the study of the relation between the tricarboxylic acid cycle as it operates in renal energy metabolism and its function in organic acid excretion renal regulation of acid base balance, and calcium excretion

So from several standpoints the tricarboxylic acid cycle in the kidney presents the biologist and clinician with a variety of interesting and important problems, and it is to some of these that we must now turn our attention

Citrate and α Ketoglutarate

Although it has been known for some time that citric acid is a constituent of normal human urine[3] and although urinary citrate excretion is known to vary under many circumstances there has appeared no definitive study, either by micropuncture or clearance techniques, of its mechanism of excretion by the kidney A few determinations of citrate/creatinine clearance ratios in the dog[4] indicate that it is filtered and reabsorbed in this species

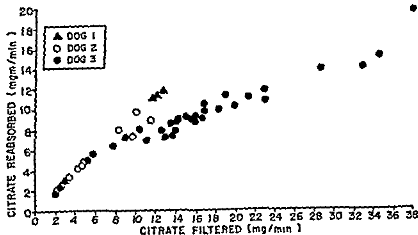


Figure 15 Data from three dogs showing the relationship between citrate filtration and reabsorption. No citrate Tm was observed in these animals at plasma citrate levels below those that produce ventricular fibrillation From Keyl (5)

Keyl[5] has shown that citrate is filterable from plasma over a wide range of concentrations and has infused normal dogs with sodium citrate up to their level of cardiac toxicity In Figure 15 the results of these initial studies on three dogs are shown It can be seen here that citrate is filtered through the glomeruli and reabsorbed by the tubules Unlike glucose or phosphate, however, no Tm for citrate has been demonstrable over the rather wide range of filtered loads achieved in these animals Therefore, if a maximal rate of citrate reabsorption does exist in the dog kidney, it cannot be shown at plasma levels below the point of cardiac toxicity

Effect of acid base balance—In 1931 Ostberg[6] first demonstrated that urinary citrate excretion rises in alkalosis This observation was confirmed five years later by Sherman, Mandel, and Smith[7] and has since been repeatedly observed by others [8,9] The phenomenon is demonstrable in metabolic alkalosis produced by infusion of sodium bicarbonate However, Cooke and his associates[8] have shown that total organic acid excretion rises much more sharply in rats when they are given potassium bicarbonate after a preliminary period of potassium depletion The results of one of their experiments are illustrated in Figure 16

Potassium depletion was effected by feeding a low potassium diet coupled with the administration of desoxycorticosterone acetate (to facilitate the renal loss of potassium) Following the depletion period potassium bicarbonate was given intraperitoneally in a dose of 6 mEq/Kg/day for 5 days The data of Figure 16 show that this treatment produced a marked augmentation in organic acid excretion, most of which was citrate It is important to note that this organic aciduria was accomplished without much elevation in bicarbonate output or rise in urine pH It should also be noted that it was accompanied by a decrease in chloride excretion Here is an indication that the excretion of organic acid anion may be acting to conserve the fixed anion chloride

In a study confirming and extending these findings, Evans, MacIntyre, MacPherson, and Milne[9] observed the effects of both metabolic and respiratory alkalosis on organic acid excretion in man and of metabolic alkalosis in the rat They showed that

organic aciduria occurs in man in response to either bicarbonate infusion or potassium depletion and that it does not necessarily require alkalinization of the urine Figure 17 shows one of their experiments on a man with primary aldosteronism with potassium

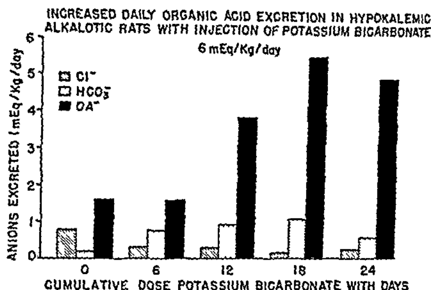


Figure 16 Observations on hypokalemic rats illustrating the increased daily organic acid excretion following the injection of potassium bicarbonate From Cooke *et al* (8)

depletion The administration of potassium chloride to this man produced a marked retention of potassium and an augmentation of organic acid excretion This was mostly citric acid, but there was in addition a measurable rise in excretion of *a* ketoglutarate These two acids of the tricarboxylic acid cycle always appear to comprise the organic acids that are excreted in metabolic alkalosis both in man[9] and rat[9 10]

The study in Figure 17 further shows quite clearly that an alkaline urine is not an absolute requisite of the organic aciduria, indeed, in this experiment it occurred in the presence of a falling urine pH And finally it is important to record that there is no change in plasma level of these acids during their augmented rate of excretion Thus this process must be the result of decreased

tubular reabsorption or an increased tubular excretion of citrate and α ketoglutarate rather than a change in their rate of glom erular filtration

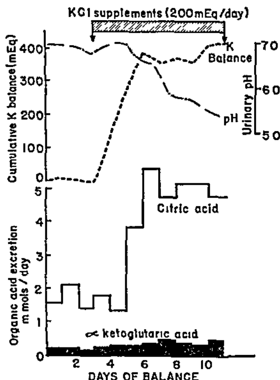


Figure 17 Observations on a patient with primary aldosteronism show ing the increased excretion of citric and α ketoglutaric acids following the administration of KCl 200 mEq/day during the interval indicated by the cross hatched bar at the top of the figure Note that the increased excretion of organic acids occurred despite a fall in urinary pH Discussion in text From Evans MacIntyre McPherson and Milne (9)

Evans *et al* [9] went on to observe in their human subjects that there was an even greater increase in organic acid excretion dur ing respiratory alkalosis caused by hyperventilation than they had found in metabolic alkalosis Again citric and α ketoglutaric acids were the main components of the respiratory response But

in addition, there was a fraction in all cases that was neither of these acids In one subject this turned out to be lactate and pyruvate However, in this subject the plasma level of these two acids was elevated, whereas in none of the respiratory alkalotics was there plasma elevation of citrate or α ketoglutarate Thus the increased excretion of pyruvate and lactate seen in the one subject can probably be explained on the basis of elevated filtered load of these two acids Such an explanation, however, cannot account for the higher levels of excretion of citrate and α ketoglutarate seen in the respiratory alkalosis

In *metabolic* alkalosis produced by the infusion of sodium bicarbonate there are elevated plasma pCO_2 and HCO_3 with accompanying bicarbonaturia and elevated urinary pH On the other hand, in *respiratory* alkalosis produced by prolonged hyper ventilation, while there is also bicarbonaturia and high urine pH, the situation in the blood is quite different, there is lowered pCO_2 and HCO_3 Yet in both conditions (the respiratory more than the metabolic) increased quantities of citrate and α ketoglutarate appear in the urine without measurable change in their plasma levels

What is the common denominator in these conditions, and why is the organic acid response greater after hyperventilation than after bicarbonate infusion or potassium depletion? It may be that there is a common change in intracellular pH in all these circumstances Potassium depletion is known to reduce the bi carbonate content of renal cortex slices[11] and thus in all likeli hood the intracellular pH In addition, it reduces the capacity to reabsorb bicarbonate in the kidney [12] In repletion with either potassium chloride or bicarbonate, urinary organic acid excretion rises With potassium chloride it may occur with a fall in urine pH, with the bicarbonate salt there may be a slight rise or no change at all in pH Thus urine pH does not seem to be directly related, but in both cases intracellular potassium concentration increases with an almost certain rise in intracellular bicarbonate and pH

Stern, Ochoa, and Lynen[13] have shown that the 'condensing enzyme' system in tissues that synthesize citrate from oxaloacetate and acetyl coenzyme A is extremely pH sensitive, more citrate

being formed as the pH rises Were changes in intracellular pH, coincident with a rising intracellular potassium concentration, to cause such an increased rate of citrate synthesis *in vivo*, one could then assume that citrate, and its oxidation product α ketoglutarate, could be present in high enough concentration to diffuse out of the cells to the point of lower concentration in the luminal urine In strong support of such an hypothesis is the report of Milne, Scribner and Crawford [14] They have observed in normal rats that the administration of potassium bicarbonate causes a rise in renal tissue citrate within twenty minutes to levels comparable with those seen in kidneys from rats treated with fluoroacetate This is, indeed, a striking and rapid rise in intracellular citrate and most germane to the problem of organic aciduria seen in this condition The fact that the organic aciduria occurs without any change in plasma organic acid level in the presence of a sharp rise in renal tissue citrate makes tubular secretion of citrate a most likely possibility

Although never directly demonstrated this likelihood of tubular secretion of citrate is further strengthened by the studies of Orten and Smith[15] published in 1937 They infused dogs with sodium salts of a number of organic acids and noted that with some of them there was a marked rise in urinary citrate excretion (Fig 18) This response was most evident with malic and succinic acids Although not shown in the figure, the authors also report large increase in citrate excretion following maleic acid and the succinic dehydrogenase inhibitor, malonate Although bicarbonate itself caused a mild increase in citrate excretion this response was insignificant in comparison to what occurred with similar rise in urinary pH during infusion of malate or succinate Orten and Smith stressed in their paper that rising pH was not the primary or even necessary correlate of the citraturia they saw with substrate infusions or that was seen in alkalosis This conclusion is strengthened by the data of Evans *et al* discussed above Although the citraturia seen during infusion of these precursors could be the result of a depressed citrate reabsorption these data could also mean that citrate is synthesized by the tubules from other acids of the tricarboxylic acid cycle and then secreted into the urine

This conclusion drawn from the work of Orten and Smith is in harmony with the subsequent studies of Krebs[16] and the recent work of Nordmann and Nordmann[17] Krebs was stimu lated by Orten and Smith s experiments repeated and extended them in rats and rabbits and explained them in terms of the tricarboxylic acid cycle that was just coming to definitive form

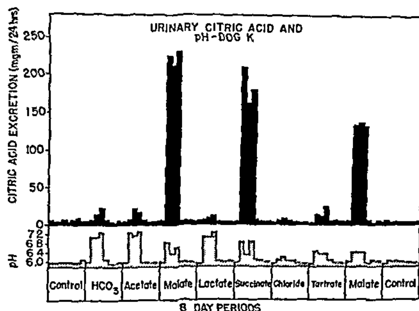


Figure 18 Observations on urinary citric acid excretion and pH in a dog following periods of intravenous administration of b carbonate and a number of organic acids The findings are discussed in the text. From Orten and Smith (15)

in his laboratory His concept of the cycle reactions was not avail able to Orten and Smith when they did their experiments a few years earlier, and therefore they were unable to explain their results in terms of his scheme

Nordmann and Nordmann have presented an elegant paper chromatographic technique for identifying and measuring all the cycle acids in urine and using this have studied organic acid ex cretion during cycle substrate infusions In both rat and man citrate and α ketoglutarate excretion rises after infusion of

α ketoglutarate, succinate, and L-malate These investigators see the kidney as the major site of citrate metabolism in the body Although it is questionable whether it is the 'major site," the kidney does have a brisk uptake of citrate This fact was shown first by Martensson[18] using the perfused kidney and has more recently been shown again by Herndon and Freeman[19] using renal arterio-venous extraction on the intact kidney *in situ*

The lack of a relationship in all these studies between urine pH and organic acid excretion makes it appear unlikely that the citrate so formed within the cell diffuses in accordance with a pH gradient This conclusion is strengthened by the fact that the molecular properties of citrate and α ketoglutarate are very unlike those of substances excreted by a diffusion mechanism As Milne and colleagues[14] point out, their molecules contain many

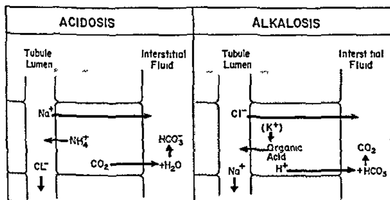


Figure 19 A hypothetical scheme visualizing an anion exchange between organic acid and chloride acting as an anion conservation mechanism in alkalosis comparable to the cation conservation exchange mechanism in acidosis involving ammonia hydrogen and fixed cation Discussion in the text. From Cooke *et al* (8)

polar groups, thus rendering the free acid relatively lipoid insoluble and *less diffusible* across biological membranes

Cooke[8] has suggested that organic acids are secreted by the tubules He postulates that this mechanism might function, according to the scheme shown in Figure 19, as an anion exchange

mechanism in alkalosis Such a scheme visualizes the conservation of the chloride anion in alkalosis as analogous to the conservation of the fixed cation in acidosis Thus in alkalosis *there is an anion exchange* between Cl^- and organic acids (OA^-) just as in acidosis there is a cation exchange between Na^+ and H^+ The existence of such an organic acid anion secretory mechanism for citrate and α ketoglutarate remains to be demonstrated directly But the likelihood of its existence is a strong one on the basis of the studies of Orten and Smith,[15] Krebs,[16] and Nordmann and Nordmann[17] discussed above In an analogous situation the tubular secretion of *malic acid* during infusion of citrate, α ketoglutarate, or succinate has been *positively demonstrated in the dog*[20 21] *This* phenomenon will be discussed in detail below Whether organic acid excretion functions as an anion exchange system conserving fixed anion in alkalosis remains to be seen, nevertheless, it is an interesting teleological suggestion

Citrate excretion and the menstrual cycle—Variations in citric acid excretion coinciding with the phases of the menstrual cycle have been observed by Shorr, Bernheim, and Taussky[22] Citrate excretion is lowest during menstruation, then rises in the immediate postmenstrual period until it shows a dip for a day or two in the mid period of the cycle This is followed by a rise to even higher levels of excretion than before, and this is sustained until the sharp fall just before the onset of the next menstruation

Evidence supporting the hormonal nature of this cyclic variation in citrate excretion derives from hormone substitution studies Estradiol given to two amenorrheic females caused significant elevation of citrate excretion and a return to normal rates when treatment was stopped, the reverse effect was observed when testosterone was given to a man with pituitary hypogonadism Similar results were obtained when this steroid was administered to an amenorrheic female

Such results indicate a sex hormone control over citrate excretion both *in* the male and female, estrogens and perhaps progesterone increasing and testosterone decreasing the rate of citrate excretion It is not known whether these effects represent altered glomerular filtration, tubular transport of citrate, or

both Clearance studies will have to be performed to answe
problem Furthermore, it is not clear whether the horm
affect the renal handling of citrate directly or whether they
duce changes in acid base equilibrium which then secon
affect the renal citrate mechanisms

Relation to calcium excretion—Except in certain typ
severe acidosis the excretion of citrate and calcium generall
parallel Citrate excretion is low in hypoparathyroidism,[23]
along with calcium, it rises after treatment with parathyroi
tract or vitamin D preparations

Under certain conditions where rats are given a diet lo
phosphorous but high in calcium, the chronic administrati
the carbonic anhydrase inhibitor, acetazoleamide will caus
formation of renal calculi in the tubules beginning at the co
medullary junction Harrison and Harrison who observed
phenomenon[24] also noted that acetazoleamide completely inl
the renal excretion of citrate even though the urine bec
alkaline Since plasma citrate is unchanged after acetazolea
this drug probably causes an intracellular acidosis which in
reduces the cellular synthesis of citrate and thus its excre
Although not reported in the literature to the author s kı
edge, it would be of great interest to measure renal tissue ci
after acetazoleamide treatment For if the formulation, disc
earlier is correct, acetazoleamide, like potassium deficienc
a general metabolic acidosis should lower the intracellular
and thus the capacity of the tubule cells to synthesize ci
Such measurements of renal tissue citrate after acetazolea
(and in potassium deficiency) would afford a vital counterpa
the observed high level of citrate in the kidney of the rat
receiving $KHCO_3$

Thus citrate excretion is a complex process which has been only superficially studied Renal tissue citrate levels and careful experiments with clearance and stop flow techniques are required to elucidate the contributions of glomerular filtration and tubular function in citrate excretion under a variety of circumstances These should include studies during acid base changes produced by infusions of acids and alkali, acetazoleamide, hyperventilation and potassium depletion The clarification of a possible tubular synthesis and secretion of citrate is a needed research And finally a study of sex steroids and parathyroid hormones would add much useful information in the understanding of the mechanism of citrate excretion

As with phosphate, amino acids, and glucose, we are dealing with an important metabolite when we consider citrate, and from the discussion that follows we will see that the cellular metab olism of such substrates places major conditions on our approach to the study of their excretion and transport

Tubular Synthesis and Transport of Malic Acid During Infusion of Cycle Acids

In 1953 Craig, Miller, and Woodward[20] reported that during the infusion of either α ketoglutarate or succinate in dogs there was filtration and net tubular secretion of malic acid in the urine Under these conditions the addition to the infusion of the suc cinic dehydrogenase inhibitor, malonic acid, caused an abrupt drop in malate excretion presumably by inhibiting its tubular secretion In addition, these investigators showed that after mal onate there was still a net filtration and reabsorption of malic acid Thus for this organic acid there appeared to be combined processes of glomerular filtration, tubular secretion and reabsorp tion operating in its overall mechanism of excretion

These provocative observations have recently been repeated and extended by Vishwakarma and Lotspeich[21] In Figure 20 are summarized the results of their studies on malic acid excre tion in the dog during infusion of three substrates in the cycle namely, citrate, α ketoglutarate, and succinate During infusion of the sodium salts of any one of these three acids there is an ele vation of both plasma level and urinary excretion of malic acid

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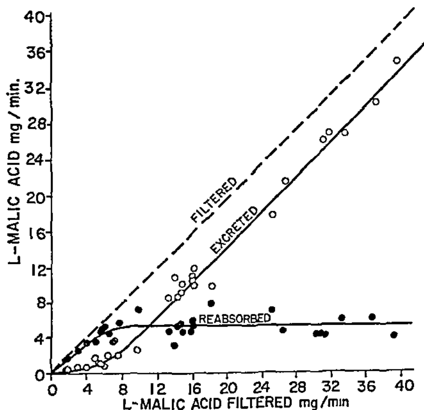


Figure 21 The relationship between l malic acid filtered excreted and reabsorbed in the dog These observations are taken from experiments during the infusion of sodium l malate Note that under these conditions there is a Tm malate which is quite constant over a wide range of filtered malate loads From Vishwakarma and Lotspeich (21)

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These experiments on the dog showing malate secretion dur ing infusion of citrate α ketoglutarate or succinate have been partially confirmed in the chicken Vishwakarma[25] has shown that succinate infusion in this species also leads to malate secre tion by the renal tubules

Malate excretion during infusion of L malate or fumarate— It next became of interest to study the pattern of malate excretion in the dog during infusion of fumarate or malate itself both of which appear after succinic dehydrogenase in the cycle Malic acid is freely filterable over a wide range of plasma levels and when one studies its mechanism of excretion during infusion of sodium L-malate it can be observed that L malate is filtered and undergoes a net tubular reabsorption In contrast to the experi ments with citrate α ketoglutarate or succinate no tubular se cretion was seen The data[21] summarized in Figure 21 reveal that malate exhibits a reabsorptive Tm at filtered loads above 6 to 8 mg per minute In this respect therefore malate reab- sorption *during L malate infusion* resembles reabsorptive mech anisms for glucose sulfate phosphate and amino acids

Subsequent experiments on malate excretion during fumarate infusion revealed an interesting difference in the capacity of the tubules to reabsorb malate under these circumstances Table III shows experiments illustrating this difference During infusion of both substrates marked elevation of plasma malic levels were observed With both substrates there was always glomerular fil tration with net reabsorption In contrast however even though the filtered load of malate was nearly identical in the two experi ments the reabsorptive rate of malate was six to seven times higher during the infusion of fumarate than it was during the infusion of L malate itself Subsequent studies designed to eluci date this observation showed that fumarate in very small amounts exerts a catalytic effect on malate reabsorption For instance as is seen in Figure 2? the addition of as little as one sixtieth the molar amount of fumarate to an L malate infusion raises the rate of malate reabsorption three or four fold even though the filtered load of malate does not show a proportional rise

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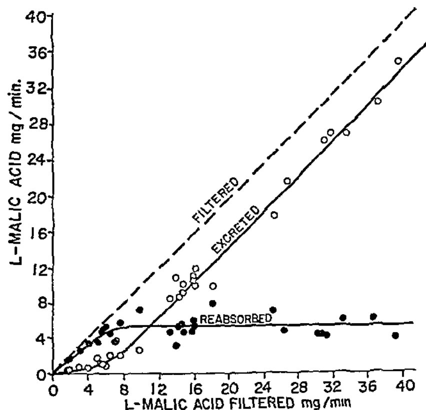


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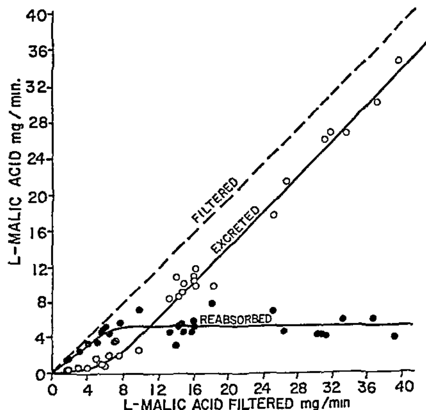


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TABLE III

EXPERIMENTS ON THE SAME DOG SHOWING HIGHER RATE OF MALATE REABSORPTION DURING FUMARATE THAN DURING L-MALATE INFUSION[31]

Total Concurrent Time	Glomerular Filtration Rate	L Malic Acid			
		Plasma	Filtered	Excreted	Reabsorbed
min	ml/min	mg/ml	mg/min	mg/min	mg/min
Fumarate Infusion					
30–40	111 6	336	37 5	7 92	29 6
40–50	111 4	331	36 9	9 00	27 9
50–60	109 0	327	35 7	9 00	26 7
L Malate Infusion					
30–40	93 0	420	39 0	35 0	4 0
40–50	93 4	390	36 5	30 4	6 1
50–60	98 3	336	33 0	27 1	5 9

The nature of this catalytic effect of fumarate on malate reabsorption is not clear on the basis of studies so far It may represent a specific facilitation in the carrier system for malate across the tubule based on the similarity of molecular structure of the two acids We have already seen that malate can be both secreted and reabsorbed by the tubules Were fumarate to inhibit a secretory limb in this bidirectional flux, there would be measured an increased net reabsorption of malate This seems an unlikely possibility, however, in view of the magnitude of malate reabsorption during fumarate infusion Here the reabsorptive rate is some six to seven times greater than the entire malate transport during malate infusion Thus the postulated secretory element would have to be greater than the 4 to 8 mg /min reabsorptive element in order to have its inhibition produce a net reabsorptive increase some 6 to 7 times this amount For these reasons a direct *stimulatory* effect of fumarate on malate reabsorption seems at present a more likely explanation of the observed phenomenon

One must also consider the possibility that fumarate is catalytically increasing the *utilization* of malate by the tubule cells When one measures the net 'reabsorption" or 'secretion' of a

substance by clearance techniques, one merely observes that more or less of the substance appears in the urine than was filtered through the glomeruli in a unit of time Therefore, one cannot say with certainty that these observations represent true reabsorption or secretion. This is especially the case with metabolites such as those under discussion.

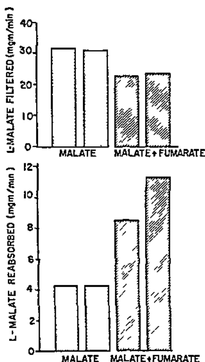


Figure 22 Experiment on the dog illustrating the "catalytic" effects of fumarate on l malate reabsorption Discussion in text. From Vishwakarma and Lotspeich (21)

A measured net "reabsorption," for instance, could result if the tubules "utilized" a metabolite presented to their luminal cell borders by the glomerular filtrate. Thus catalytic amounts of fumarate could accelerate the renal "utilization" of malate, or its reabsorption, or both. Simultaneous measurements of reabsorption,

using clearance techniques, and utilization by arteriovenous dif ference studies are required to differentiate these two processes

As was mentioned earlier, Cohen[2] has recently performed such studies with a series of oxidative substrates including aceto acetate, lactate, pyruvate, and α ketoglutarate Acetoacetate has a high rate of reabsorption and a low rate of utilization pyruvate and α ketoglutarate, on the other hand, show low rates of net reabsorption but high rates of utilization Such studies have not yet appeared for malate but will be helpful in locating whether the fumarate effect is on malate reabsorption, utilization, or both

These experiments with malic acid during infusion of dif ferent tricarboxylic acid cycle substrates indicate that the cycle functions in the kidney not only in its oxidative metabolism but also in the transport of at least one of its own intermediates malic acid The citrate experiments of Orten and Smith,[15] the confirmatory experiments of Krebs [16] and the more recent studies of malate infusion in the rat[17] strongly support the theory that in addition to synthesizing and secreting malate from cycle pre cursors, the tubules can likewise make citrate from malate and succinate and secrete it into the urine These studies have been discussed in relation to the phenomenon of organic acid excretion in alkalosis where citrate is the major ion Whether the secretion of malate represents a related process remains to be seen

Comparison of D and L malic acid transport in the kidney— From a metabolic standpoint L malate is the natural isomer that is oxidized in the cycle to oxaloacetate by the DPN linked malic dehydrogenase enzyme system in kidney and certain other tissues Preliminary experiments designed to study the mechanism of excretion of malate during infusion of the D isomer in the dog produced equivocal results Sometimes there was net reabsorp tion at others, net secretion It was therefore, decided to perform similar experiments in the chicken in an attempt to clarify the problem

The chicken has a renal portal circulation Sperber[18] has shown that a substance administered into one leg vein of this species will perfuse the interstitial space around the renal tubules before reaching the general systemic venous return and thence

the glomerular vessels Thus if one infuses a substance into one saphenous vein of the chicken and collects urine simultaneously from both ureters separately, a higher rate of excretion of that substance from the kidney of the infused side means that the substance has undergone tubular excretion conversely, no difference in rate of excretion on the two sides is interpreted to mean that no tubular secretion has occurred Experiments of this type have been performed by Vishwakarma[23] with the D and L isomers of malic acid in the chicken and the results are summarized in Figures 23 and 24

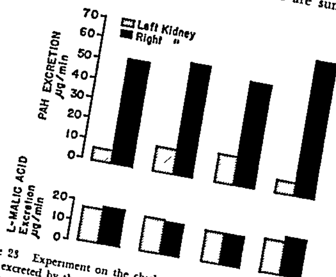


Figure 23 Experiment on the chicken illustrating the fact that malic acid is not excreted by the tubules in this species during the infusion of sodium l malate Black bar of each pair represents right kidney on the side of the infusion the cross hatched bar the left kidney on the side opposite the infusion The upper set of bars show the high rate of PAH excretion by the tubules on the infused side indicating that the renal portal circulation was open as discussed in the text From Vishwakarma (unpublished observations in the author's laboratory)

Sperber has emphasized the presence of a valve in the renal portal circulation in the chicken which when open can cause a bipass of this circulation When this happens, the tubules are not perfused Since p-aminohippurate (PAH) is known to be

may be able to synthesize citrate during succinate or malate infusion and secrete it into the urine in the dog Without any change in their plasma levels the excretion of citrate and to a lesser extent α ketoglutarate changes from a low rate in metabolic acidosis to a high rate in either metabolic or respiratory alkalosis This phenomenon is best seen during potassium repletion of potassium-depleted rats or during primary aldosteronism and hyperventilation in man The increased rate of citrate and α ketoglutarate excretion seen in *these instances does not necessarily* depend on the excretion of an alkaline urine but is more likely related to changes in intracellular citrate and α ketoglutarate synthesis at higher intracellular pH The possibility that organic acid secretion acts as a fixed anion conservation mechanism in alkalosis was discussed and needs additional work

Changes in citrate excretion independent of plasma citrate concentration also occur as a result of sex hormone and acetazoleamide administration The sex hormone effects have not been studied carefully the acetazoleamide inhibition of citrate excretion is best understood again as an intracellular acidosis with consequent effects on citrate synthesis and secretion One important function of citrate in urine is its solubilization of calcium with which it forms a chelate and thus prevents renal calcinosis

One can demonstrate quite clearly the tubular secretion of malic acid during infusion of citrate α ketoglutarate or succinate all of which are precursors of malate in the tricarboxylic acid cycle In contrast during infusion of malate or fumarate tubular secretion of malic acid cannot be demonstrated It is filtered through the glomeruli and shows a net tubular reabsorption Again during L malate infusion its reabsorptive rate is markedly increased upon addition to the infusion of catalytic amounts of fumarate The mechanism of this interesting effect is not clear at present In the chicken malate is synthesized from succinate and secreted by the tubules as it is in the dog

Also in the chicken it has been shown that there is a striking difference in the excretion of the natural and unnatural forms of malic acid. The D form is secreted by the tubules at a high

rate while the L form is not. And this secretory mechanism is functionally separate from both those for other organic acids and the strong organic bases

These studies were discussed in the light of a close relation between the tricarboxylic acid cycle as an oxidative mechanism in cells and the synthesis and transport of the cycle's own intermediates in the kidney tubule The field of renal utilization and excretion of oxidative substrates is a new one and offers great promise not only for our understanding of basic transport processes of organic acids but for the whole problem of renal regulation of acid base balance and renal energy metabolism as it relates to the synthetic and excretory activities of the kidney

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V

THE SYNTHESIS AND SECRETION OF AMMONIA

EACH DAY the normal individual produces large quantities of acid as by products of metabolism The weaker carbonic acid is converted to carbon dioxide and excreted by the lungs, but the stronger, non volatile acids, such as phosphoric, sulfuric, and β hydroxybutyric, must be excreted by the kidneys A certain proportion of this acid load is excreted as buffer acids in the free titratable form However, within the confines of urinary pH, the quantity of strong acid that can be excreted in this way is limited Thus if there were no other mechanism for excreting strong acid anions without the concomitant loss of fixed cations, there would be an intolerable drain on this cation reserve in the body Fortunately such an additional mechanism does exist the tubule cells produce and secrete ammonia and absorb the fixed cation in return In this function the kidney plays a major role in the regulation of the acid base balance of the body

During the course of each day the equivalent of 300 to 500 ml of 0 1 N acid may be lost in the urine in the form of ammonia However, we know that this represents only a part of the potential capacity to excrete acid by this means For instance, in diabetic acidosis the ammonia secretory mechanism undergoes an adaptive hypertrophy to the extent that it can excrete ten times the amount lost under normal conditions This phenomenon is illustrated in Figure 25 The experiment of Sartorius Roemmelt, and Pitts[1] shows the changes in ammonia, titratable acid, and sodium before, during and after a period of experimental metabolic acidosis in man By days ten and eleven, at the height of the acidosis the fixed cation sodium, which had

initially taken almost the entire acid load, was almost entirely replaced by amomnia and to a lesser extent by titratable acid.

The synthesis and secretion of ammonia by the kidney not only represents one of its most important but also one of its most intellectually intriguing metabolic activities. Its understanding requires the description of at least four separate though interrelated processes: (1) The source of the ammonia; (2) the biochemical reactions and enzymes involved in its synthesis; (3) the site, mechanism, and regulation of its diffusion into the tubular urine; and (4) the mechanism of the adaptive increase in its secretion. We will consider each of these processes in turn.

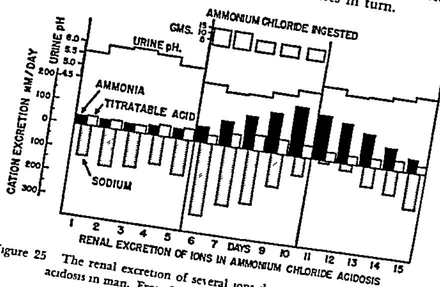


Figure 25 The renal excretion of several ions during ammonium chloride acidosis in man. From Sartorius, Roemmelt, and Pitts (1)

The Source of Urinary Ammonia

Nash and Benedict[2] first showed conclusively in 1921 that ammonia is formed by the kidney from some precursor in the blood. Some thought that the ammonia came from blood urea,[3,4] others from amino acids,[5] and still others from the amide nitrogen of plasma proteins.[6] In 1943 Van Slyke and his associates[7] studied the renal extraction of various nitrogenous constituents of plasma in the dog with an explanted kidney. Their studies showed that

in the acidotic animal some 60 per cent of urinary ammonia could be accounted for as coming from plasma *glutamine* and some 40 per cent from plasma *amino acid nitrogen*

Subsequent studies have shown that the infusion of glutamine, asparagine, and certain amino acids in normal[8] and acidotic dogs[9,10] elevates the rate of ammonia excretion in the urine In 1941 Bliss[9] showed that this effect could follow the injection of both natural and unnatural isomers of certain amino acids, especially alanine and leucine in acidotic dogs The subsequent studies of Lotspeich and Pitts[10] and Kamin and Handler[8] further demonstrated that some amino acids do and others do not enhance ammonia excretion when their plasma levels are elevated

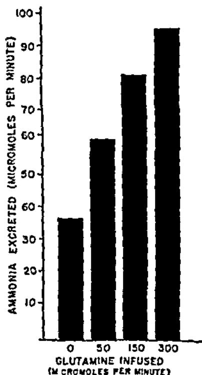


Figure 26 The relationship between ammonia excretion and rate of glutamine infusion in the dog (Unpublished data from the author's laboratory)

by infusion In general the amino acids fall into three groups of descending capacity in this regard L glutamine, L-asparagine, L- and D alanine, and L histidine are highly effective, L aspartic acid, glycine L leucine, L methionine, and L-cysteine are moderately effective, and L-glutamic, L lysine, and L-arginine are completely ineffective in stimulating ammonia excretion The relation between the administration of certain amino acids and

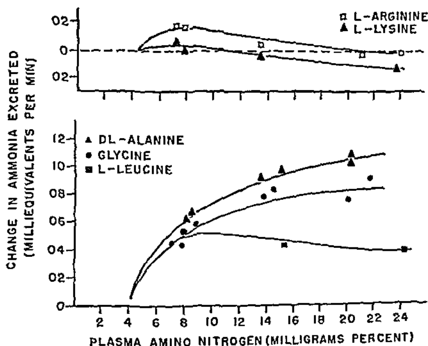
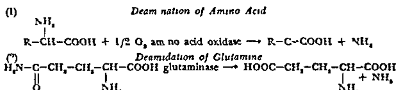


Figure 27 Relationship between plasma amino nitrogen and rate of ammonia excretion during infusion of several different amino acids From Lotspeich and Pitts (10)

glutamine and the changing rate of urinary ammonia excretion is shown in Figures 26 and 27 The ability of glutamine, DL alanine, glycine, and to a lesser extent L leucine to increase the rate of ammonia excretion is apparent from these data In contrast, however, L-arginine and L-lysine are *completely* without effect in this function

The Biochemical Reactions in Ammonia Synthesis

If one concludes that urinary ammonia derives from amino acids and their amides it is necessary to demonstrate the appropri ate enzymes and reactions whereby the ammonia from these pre cursors can be removed and made available for secretion into the luminal urine It has been assumed in recent years that the am monia is produced by the straight forward *deamination* of the amino acids themselves The ammonia so produced then diffuses into the tubular fluid The enzymes glutaminase[11] and asparag inase[12] are present in kidney as well as at least three amino acid deaminases *glycine oxidase,*[13] *D amino acid oxidase*[14] and *L amino acid oxidase*[13,15] The reaction whereby ammonia is pro duced by these mechanisms may be written as follows



In their studies on amino acid infusion Lotspeich and Pitts[10] noted a correlation between the capacity of an amino acid to accelerate ammonia excretion and the rate at which that same amino acid was enzymatically deaminated They assumed a simple oxidative deamination leading to ammonia synthesis from amino acids and it seemed logical that any one amino acid s capacity to act as an ammonia precursor should be limited by its rate of oxida tive deamination However, this theory was effectively criticized subsequently by Kamin and Handler[8] who showed that the corre lation noted by Lotspeich and Pitts[10] was not absolute in a larger series of amino acids Furthermore they pointed out that L-amino oxidase as such is not present to any measurable degree in dog kidney In view of the more recent work on intermediary metabo- lism of amino acids, the criticism of a simple deamination theory of ammonia synthesis appears justified and alternative possibilities *such as a transamination hypothesis must be considered*

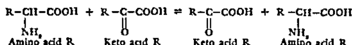
The present concept centers largely on glutamine as the main source of urinary ammonia The assumption is made that

In addition, it is necessary to discuss the relation of the tricarboxylic acid cycle to this process Through its intermediates, α ketoglutarate and oxaloacetate, this cycle is in close dynamic equilibrium with amino acid turnover on the one hand and glutamine metabolism on the other through the complex reactions of transamination and deamidation For this reason the available supply of tricarboxylic acid cycle intermediates in the course of aerobic respiration in kidney could vitally affect the capacity of the kidney to produce ammonia from glutamine

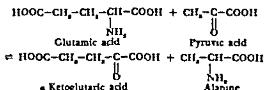
We must therefore, turn at this point to a somewhat detailed discussion of amino acid transamination and deamination and the tricarboxylic acid cycle as they relate to the intracellular assimilation of glutamine

Transamination and Deamidation

Since the pioneer work of Braunstein and Kritzman[17] in 1937, it has been apparent that there are intermolecular transfers of amino groups from amino to keto acids in certain tissues In this process a new amino acid and new keto acid are formed This type of reaction has been termed transamination and can be written in general terms as



As an example of this reaction one can cite the transamination between glutamic and pyruvic acids leading to the formation of alanine and α ketoglutaric acid

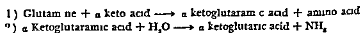


Braunstein and Kritzman's work has revealed that some sixteen amino acids, when incubated with muscle tissue, are capable of transaminations of this type Subsequent work has shown that the enzymes called *transaminases* are present in a variety of tissues

including kidney Several keto acids can act as acceptors of the amino group of glutamic acid and conversely several amino acids other than glutamic acid readily yield their amino groups to α ketoglutaric Two enzyme systems carrying out transamination reactions have been purified both contain pyridoxal phosphate They are (1) The *aspartic glutamic transaminase* catalyzing the reaction—α ketoglutaric acid + aspartic acid ⇌ oxalacetic acid + L-glutamic acid and (2) the *alanine glutamic transaminase* cata lyzing the reaction—α ketoglutaric acid + L alanine ⇌ pyruvic acid + L glutamic acid

One can see in both these reactions the key position of α ketoglutaric and L glutamic acids both reactants are always involved These transamination systems represent a major central pathway in tissue metabolism for the synthesis and intercon version of amino acids It is easy to see therefore that this vast pool of amino acid metabolism could easily form a route over which amino groups could be passed in the synthesis of glutamine

In addition to transamination reactions of the type just dis cussed there are also *transamination deamidation* reactions in volving amino acid amides These have been described and sum marized by Meister[18,19] In the case of glutamine it has been shown that certain keto acids stimulate the simultaneous and coupled transamination of the amino group to the keto acid and the deamidation of the amide group with formation of free am monia Meister has shown clearly that these reactions occur in two steps as follows

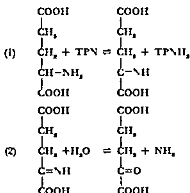

1) Glutamine + α keto acid ⟶ α ketoglutaramic acid + amino acid
2) α Ketoglutaramic acid + H_2O ⟶ α ketoglutaric acid + NH_3

Here the first step is transamination with formation of the new amino acid and keto analog of glutamine then in the second step the deamidation of α ketoglutaramic acid yields α ketoglutaric acid and free ammonia

The analogous reaction with asparagine forms α ketosuc cinamate in reaction (1) which then is deamidated to oxalacetate and ammonia in reaction (2) The enzymes involved in these reactions also contain pyridoxal (vitamin B_6) as was the case in the transaminases discussed above

Relation to the Tricarboxylic Acid Cycle and Glutamic Acid Dehydrogenase

These transamination-deamidation reactions all involve α ketoglutaric and glutamic acids Glutamic acid in turn can be oxidatively deaminated to yield ammonia and α ketoglutaric acid by an enzyme called *glutamic dehydrogenase* which utilizes coenzyme I or II With coenzyme II (TPN) this reaction occurs in two steps



In the formulation of Duda and Handler[16] these reactions would play an intermediary role in the synthesis of glutamine from ammonia that originated in L-amino acids Thus if the renal production of ammonia depends on a continuing intracellular synthesis and then deamidation of glutamine, this *glutamic dehydrogenase* system with an adequate supply of oxidized TPN becomes a vital element in the mechanism Whether the oxidative deamination of glutamic acid by this enzyme system can function directly as a source of urinary ammonia is unknown at present However, from the available evidence this possibility appears unlikely

If *glutamic dehydrogenase* does play this intermediate role between L-amino acid transamination and glutamine synthesis then an active *cytochrome reductase* becomes essential in the terminal electron carrier system to maintain a supply of oxidized TPN for the glutamic acid deamination Thus for this reason, as

well, the general level of oxidative respiration in kidney affects its capacity to produce ammonia

Relation between tricarboxylic acid cycle and fate of ammonia produced by deamidation of glutamine—It is well known that substrates in oxidative metabolism can affect both the rate of ammonia production in glutamine deamidation and its final disposition in the tissue Thus pyruvate and certain other keto

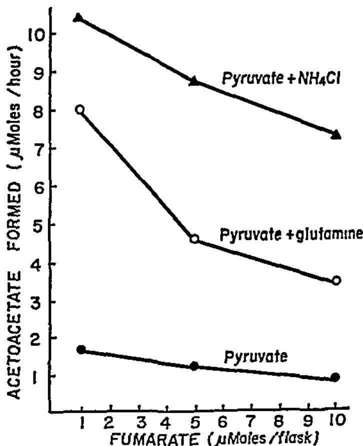


Figure 28 A comparison of the ketogenic effect of ammonium chloride and glutamine in guinea pig liver homogenate *in vitro* at various levels of fumarate For relation of these observations to the problem of ammonia production in the kidney see discussion in the text. From Lotspeich (Unpublished observations)

cids can stimulate the glutamine transamination-deamidation ystem, and α ketoglutarate can act as an acceptor for the am nonia so produced in its reductive amination to glutamate For hese reasons it is reasonable to assume that ammonia produced vithin the cell can either diffuse into the tubular urine or, in he presence of an abundant supply of α ketoglutarate, be disposed f inside the cell in forming glutamate by reductive amination To test the capacity of glutamine to participate in this reductive mination of α ketoglutarate, experiments such as those shown n Figure 28 were performed

Recknagel and Potter[20] had studied the function of ammonia tself, in the form of NH_4Cl, to carry out this reaction They used omogenates of rat liver, pyruvate was the substrate, and ace oacetate accumulation was used as a measure of the amination of ketoglutarate in the presence of NH_4Cl In this system pyruvate eads to α ketoglutarate which is then converted to glutamate This piles up and blocks the tricarboxylic acid cycle by cutting off the supply of oxalacetate for the initial condensation reaction In the absence of sufficient oxalacetate, the acetate fragments from pyruvate condense with each other to form acetoacetate The addition of fumarate regenerates a supply of oxalacetate which hen condenses with the acetate to form citrate and the acetoacetate accumulation is reduced This effect of NH_4Cl in aminat ing α ketoglutarate is seen in the large increase in acetoacetate between bottom and top curves in Figure 28 In addition, the data reveal the way in which increasing concentrations of fumarate reduce the acetoacetate production

In the middle curve it is seen that addition of glutamine to the homogenate respiring pyruvate causes almost as much acetoacetate to accumulate (at low fumarate levels) as did NH_4Cl itself Furthermore, as fumarate concentration is increased, the amount of acetoacetate that accumulates is sharply decreased in dicating that the formation of glutamate in the reductive amina tion of α ketoglutarate is likewise decreased

These data are consistent with the concept that the ammonia formed in the deamidation of glutamine is available for the re ductive amination of α ketoglutarate And to the extent that this

reaction takes place, the ammonia so produced from glutamine will not be free to diffuse into the urine Thus both the production of ammonia in the transamination deamidation systems in the kidney and its availability for diffusion into the urine depend on a number of important variables Among these may be the supply of pyruvate and other keto acids, the supply of α ketoglutarate produced in transamination reactions or in the tricarboxylic acid cycle, and the availability of oxalacetate which determines the rate at which the cycle turns For these reasons it is obvious that we must take into account the whole complex and dynamic equilibrium between glutamine (and other amides), the amino acid transamination reactions, the deamination of glutamate, and finally the tricarboxylic acid cycle when attempting a complete description of ammonia production in the kidney By way of summarizing these complex relations we have been discussing, Figures 29 and 30, taken from Meister,[19] are presented All this discussion points forcefully to the necessity of a much broader approach to the problem of the renal production of am

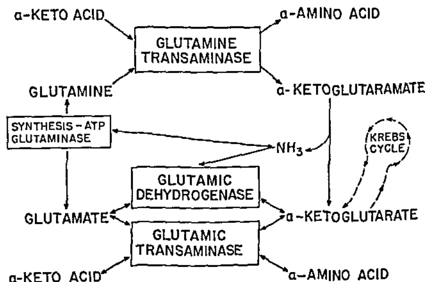


Figure 29 Metabolic interrelations between glutamine glutamate, amino acids, and the Krebs tricarboxylic acid cycle in tissues. For relation to ammonia synthesis in the kidney, see discussion in the text From Meister (19)

monia At the moment the research in this area is too narrowly focused on the problem of glutamine deamidation without enough attention to these other aspects of a very complex steady state situation

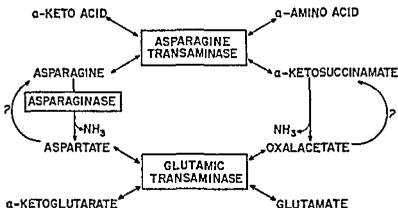


Figure 30 Interrelations of asparagine metabolism with amino acids ammonia production and intermediates of the Krebs tricarboxylic acid cycle For relation to ammonia synthesis in the kidney see discussion in the text. From Meister (19)

Mechanism of Ammonia Diffusion Into the Urine

A number of careful studies have now conclusively shown a close inverse relation between urine pH and rate of urinary ammonia excretion[21,22,23] This relation is clearly seen in Figure 31 from data presented by Pitts[21] on a dog in normal and acidotic states The capacity to excrete ammonia at a higher rate at any given pH in acidosis will be discussed in some detail below However, the fact that in both the normal and the acidotic dog there is a linear relation between ammonia excretion and urinary pH indicates that while degree of acidosis is one condition governing rate of ammonia excretion urinary pH is another and constant factor in both situations

It is visualized (Figure 32) that ammonia is synthesized in the tubule cells in the form of the free base

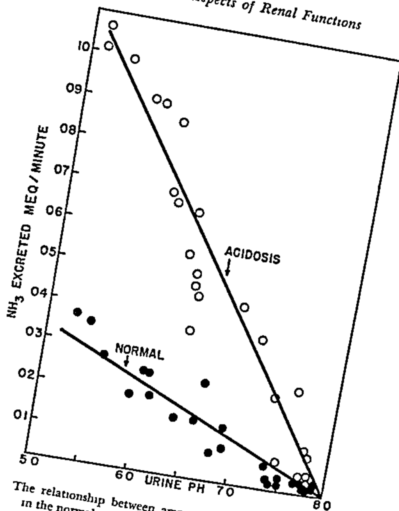


Figure 31 The relationship between ammonia excretion and urinary pH in the normal and acidotic dog From Pitts (21)

converted to the ammonium ion (NH_4^+) by combining with a hydrogen ion that has been synthesized from carbonic acid according to the scheme shown in the upper cell of the diagram The secretion of hydrogen ions sets up a pH gradient for the diffusion of the free ammonia base, and at the same time converts it to the ammonium ion in the luminal urine The close relation between hydrogen and ammonium ion secretion thus effects ammonia diffusion and cation conservation in a unique way

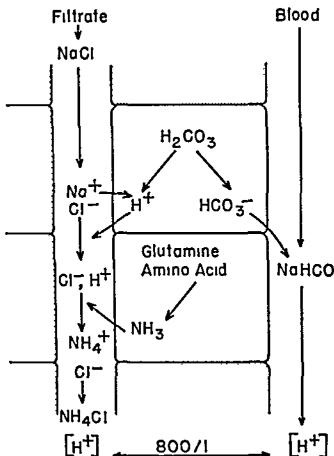


Figure 32 Scheme illustrating the source of hydrogen ions and ammonia in the kidney and their interrelations in the urinary excretion of ammonia and titratable acid. From Pitts (21)

Orloff and Berliner[23] have studied the physico-chemical aspects of the pH regulation of ammonia secretion They analyzed the factors governing the diffusion of ammonia as a weak base in terms of Jacobs'[24] general description of the mode of accumulation of weak electrolytes by cells Jacobs postulated that the distribution equilibrium of such weak electrolytes between cells and their

surrounding medium is governed by the pH of the medium, the pKa of the weak electrolyte, and the permeability of the cell membranes to the uncharged, lipid soluble species It is the unionized species of the electrolyte that is diffusible, and in the case of ammonia this is the free base (NH_3) Orloff and Berliner have shown that their experimental results conform to a theory based on these postulates and accordingly they visualize free ammonia (NH_3) as the diffusing species

Being more lipid soluble, NH_3 moves easily across the luminal membrane into the more acid tubular urine where it is trapped by H^+ ions and converted to the ammonium ion (NH_4^+) which is non diffusible, non lipid soluble and thus does not penetrate back into the cell Hence a diffusion gradient is established between cell and lumen, and the conversion of ammonia to ammonium ion serves not only to maintain the direction of the diffusion equilibrium in favor of continuous ammonia secretion but in buffering the hydrogen ions that are secreted, this ammonia diffusion facilitates the final $H^+ \rightleftharpoons Na^+$ exchange across the tubules According to this scheme the secretion of ammonia *by itself* does not conserve base it does so only in so far as it is converted to ammonium ion by the secretion of H^+ It is this process along with the buffers in the tubular urine that maintains the secretion of hydrogen ions and thus the exchange reabsorption of fixed cation

The pH gradient between cell interior and luminal urine is sufficient to facilitate maximal secretion of ammonia under normal and acidotic conditions in man and dog Thus rate of secretion of ammonia under these circumstances is not limited by diffusion but by rate of its intracellular production

This fact was forcefully demonstrated in the beautiful studies of Leonard and Orloff[75] on the regulation of ammonia excretion in the rat These investigators performed experiments which support the idea that in this species in contrast to man and dog rate of urinary ammonia excretion is governed more by its intracellular production than by urinary pH For instance, if one produces an acute acidosis in the rat with NH_4 Cl, the infusion of sodium bicarbonate causes a progressive rise in urine pH with a characteristic fall in ammonia excretion In contrast however,

[illegible] bicarbonate into rats made acidotic by [illegible] smaller decline in ammonia excretion [illegible] range The excretion of the exogenous base, atabrine, a process [illegible] affected by cellular production, drops in a similar way over the urine pH range in both types of acidosis Thus urinary pH and diffusion gradients appear constant factors for ammonia in both types of acidosis. But intracellular ammonia production differs in the two situations

In acid urines, below an indeterminate pH, somewhere be tween 6 25 and 6 00, rate of ammonia secretion is not limited by urine flow whether in water or osmotic diuresis However, at urine pH above 6 5 to 7 0, rate of ammonia secretion does become limited by urine flow[23] Under these conditions the pH gradient for diffusion is sufficiently slight and close to the pKa of ammonia itself so that volume of urine flow past the ammonia secreting cells becomes rate limiting in the establishment of the diffusion equilibrium

The Mechanism of Adaptive Changes in Rate of Ammonia Excretion

We have seen in the experiments of Figures 25 and 31 that the kidneys show a remarkable capacity to increase their rate of ammonia secretion in acidosis and that they are capable of secret ing more ammonia at any one urinary pH in acidosis than under normal conditions Since it has been so conclusively shown that rate of secretion of ammonia into acid urines is not limited by diffusion across the cell membrane but rather by rate of ammonia production within the cell, we must look to changes in the produc tion mechanisms for an explanation of the adaptive capacities shown in ammonia secretion with alterations in acid base balance

The capacity of the cell to increase its rate of ammonia pro duction could depend upon one or more variables in the reactions of this production as we have discussed them (1) The enzymes normally operating on suboptimal concentrations of substrate could be furnished more substrate (2) With a fixed amount of enzyme and substrate, other conditions indirectly affecting the

rate of the overall synthetic reaction could become rate limiting Such factors might be, as we have seen, the rate of oxidation in the tricarboxylic acid cycle, the supply of oxidized coenzymes such as TPN and the rates of turnover in the transamination-deamidation system In addition, changes in intracellular pH or concentration of ions such as K^+ or Mg^{++} could vitally alter the kinetics of the reactions involved (3) Lastly, there could be a true induced increase in one or more of the enzymes involved in ammonia synthesis Information about some of these variables is available

There has been no measurable change noted in the plasma level of glutamine[7] or amino acids[10] in going from the normal to the acidotic state Thus variation in substrate availability appears an unlikely factor in the capacity to increase ammonia output at endogenous plasma levels of glutamine and amino acids Aside from the experiment on the effects of fumarate concentration on glutamine assimilation shown in Figure 28, there have been no direct observations of effects of ions, cofactors, or tricarboxylic acid cycle variations on ammonia excretion However, changing rates of cycle activity must certainly affect the intracellular capacity to produce and thus to secrete ammonia

Glutaminase adaptation—Of all these possible variables, the one that has been most clearly shown capable of adaptive change in some species is the renal *glutaminase* system, a complex of at least three enzymes that are involved somehow in renal ammonia synthesis In a study using slices of rat renal cortex, Davies and Yudkin[26] first presented direct evidence for an adaptive change in ammonia synthesis from glutamine and several amino acids *in vitro* Rats were made chronically acidotic or alkalotic by the ingestion of HCl or $NaHCO_3$ in drinking water for at least three months Kidney slices from these animals were then incubated in the Warburg apparatus with substrate in a gas phase of 100 per cent oxygen at 37° C Glutamine or one of the amino acids was added and the ammonia liberated in 30 minutes was measured

As can be seen in Table IV, ammonia production was markedly increased in slices from acidotic and somewhat decreased in those from alkalotic rats in the presence of L-glutamine, glycine,

and L leucine There was however no significant change over control slices in either acidosis or alkalosis when L alanine or L-aspartic acid were substrates

These results suggested to the authors that there was an adaptive increase in *glutaminase* activity in the slices as a result of the chronic acidosis and no such change in alkalosis Their studies with glycine and L leucine in slices were interpreted in terms of changes in L-amino oxidase and glycine oxidase

TABLE IV

EFFECT OF CHRONIC ACIDOSIS AND ALKALOSIS ON AMMONIA PRODUCTION BY RAT KIDNEY SLICES IN VITRO

(*Taken from B M A Davies and J Yudkin Biochem J 52 407 1952*)
Ammonia Produced (μg $NH_3 - N/mg$ *dry wt/hr*)

Substrate	*Normal*	*Acidosis*	*Alkalosis*
L-Glutamine	19 0 ± 0 6	36 0 ± 3 3	15 0 ± 0 8
Glycine	0 64 ± 0 01	1 59 ± 0 07	0 43 ± 0 01
L-Leucine	1 94 ± 0 05	3 01 ± 0 03	1 02 ± 0 03
L-Aspartic Acid	3 53 ± 0 05	3 81 ± 0 06	3 73 ± 0 11
L-Alanine	0 28 ± 0 01	0 28 ± 0 01	0 28 ± 0 01

Whether it is justifiable to reach such conclusions regarding single enzymes from studies with whole tissue slices is doubtful because of the complex pathways of metabolism prevailing in such relatively well organized tissue Nevertheless the acidotic increase in ammonia production from these two amino acids is definite The exact enzyme concerned must be identified with less complex preparations

The lack of change in ammonia production by the slices with L-alanine or L-aspartic acid was interpreted as a lack of change in transaminase The authors concluded that this enzyme system was most likely responsible for L alanine and L-aspartic acid deamination Even though the results of this study must be interpreted with caution in terms of single enzymes involved (particularly in the light of Duda and Handler s studies[16]) it nevertheless represents the first clear-cut demonstration of some kind of an adaptive increase with acidosis and decrease with alkalosis in ammonia production from glutamine and certain amino acids *in vitro*

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This line of study was extended a few years later in 1955 by Rector, Seldin, and Copenhaver[27] who worked with homogenates of rat kidney and measured glutaminase activity directly The results of their studies are shown in Figures 33 and 34 Figure 33 presents a comparison of the time course of the glutaminase

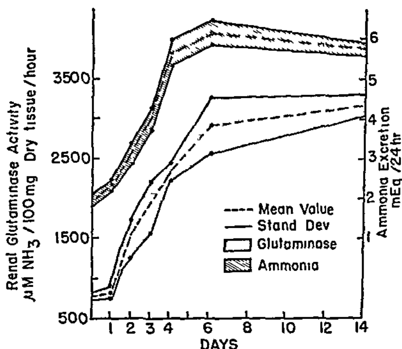


Figure 33 Observations in acidotic rats showing the parallel changes in rate of ammonia excretion and activity of glutaminase with continuous daily administration of ammonium chloride From Rector Seldin and Copenhaver (27)

adaptation and the increase in ammonia excretion during successive days of ammonium chloride administration in rats These two variables parallel one another and appear to reach maximum values around six to seven days The direct proportional relationship between glutaminase adaptation and urinary ammonia output is even more strikingly shown in the massed data of Figure 34

Combining a standard electrolyte deficient diet with ever increasing acid loads (NH_4Cl) points were obtained which fell along the straight line relating glutaminase activity to ammonia excretion during the adaptive phase Although these data do not define the exact role of glutaminase in ammonia synthesis they do emphasize that it must be *glutamine* and *glutaminase* that are most directly rate limiting in the synthesis of ammonia and thus

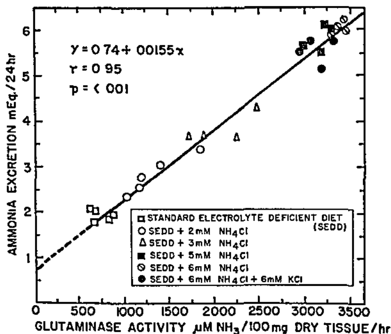


Figure 34 The direct relationship between ammonia excretion in the urine and renal glutaminase activity in the rat Experimental conditions as indicated in figure inset. From Rector Seldin and Copenhaver (27)

clarify the questions raised concerning the studies with slices These elegant data point directly to *glutaminase* as the enzyme system that adapts and thus enhances the capacity of the kidneys to excrete more and more ammonia as acidosis continues It is hoped that a similar analysis of the amino acid oxidases and transaminases will be carried out

The study of this glutaminase adaptation was carried further by Goldstein Richterich and Dearborn[28] in the guinea pig These authors had noted that in this species also renal glutaminase activity is increased in acidosis oddly enough they found an increase in alkalosis as well[29] Penetrating the problem more deeply they studied the changes in the three separate components of the glutaminase complex which they described on the basis of their own and already existing studies[19]

There are three main enzymes involved in the general metabolic assimilation of glutamine and these are all grouped under the general title glutaminase *Glutaminase I* is a hydrolyzing enzyme with a pH optimum of 7 4 and activated by phosphate *Glutaminase II* is also a hydrolyzing enzyme but differing in its pH optimum of 8 8 and its dependency on α keto acids This enzyme appears to be identical with the glutamine transaminase deamidase system described in liver by Meister[19] and discussed earlier Finally there is the *glutamine synthesizing enzyme system (glutamine synthetase)* that forms glutamine from glutamic acid and ammonia This system has an optimum pH of 7 4 in homogenates requires ATP and magnesium ions and is inhibited by p-chloromercuribenzoate

Since Goldstein *et al* did not know which components of this complex were involved in the glutaminase adaptation in the guinea pig they decided to examine the activity of each component separately in both the acidotic and alkalotic animal This analysis in kidney homogenates revealed that in acidosis the increase was limited to *Glutaminase I* while in alkalosis the activity of all three enzymes were enhanced

The change in these enzymes resembles in some respects a true induced adaptation of the type described in microorganisms by Mandelstam[30] and Monod[31] and shown to take place in mammalian liver tissue in the tryptophane peroxidase system by Knox[32] It differs however in one fundamental respect from a true enzyme induction *Glutaminase* does not appear *de novo* in the presence of substrate as does *β galactosidase* it only shows an *increased* activity in response to a change in acid base balance of the whole animal Goldstein in a separate study[33] however has attempted to analyze the glutaminase increase in terms of a true

enzyme induction and has concluded that it does meet the major requirements of such a biological phenomenon That there is an absolute increase in glutaminase I in acidotic guinea pig kidney is apparent when enzyme activity is expressed in terms of cell numbers reflected in the desoxyribosenucleic acid (DNA) con tent of the homogenates [8 33] In addition ethionine which blocks protein synthesis blocks the glutaminase I change methionine added in excess with ethionine permits it In these respects it is an enzyme induction

Whether the induction is a direct substrate effect as is the *β galactosidase* in microorganisms for example [31] or the indirect result of some other intracellular function such as pH pCO_2 or potassium concentration is not clear from available experiments. The demonstration that renal glutaminase increases under certain conditions in potassium deficiency in rats[34] without addition of acid or alkaline load to the diet makes one wonder about the role of intracellular potassium pH or concomitant changes in bicarbonate in the enzyme adaptation Such factors may, indeed, alter metabolic pathways to elevate intracellular concentration of a particular substrate This in turn could cause the change in the specific enzyme in the classical sense of an induced adaptation.

The increase in *Glutaminase I* in acidosis is easier to comprehend teleologically than the increase in all three glutamine metabolizing systems in alkalosis The two peaks in ammonia excretion (in acidosis and alkalosis) occur in the guinea pig[illegible] and rabbit [35] these two species are herbivores In the rat[illegible] and dog [21 23] both carnivores no such increase in ammonia excretion in alkalosis has been observed Perhaps this difference between carnivores and herbivores is related to the persistently greater acid ash diet in the former species Perhaps also the elevated ammonia excretion in alkalosis where it is seen is a result of the abnormally high rate of urine flow resulting from the experimental procedure used to produce the alkaline urines At such high urine pH s as we have seen ammonia excretion becomes a function of urine flow [23]

Thus in the rat and guinea pig where an increase in *glutaminase I* has been seen in chronic acidosis this enzyme adapta-

tion must be one factor at least in the continuing capacity of the kidney to excrete ammonia However, it apparently cannot be the whole story Leonard and Orloff[25] have clearly shown in the rat that there can be a fourfold increase in ammonia excretion after only twenty four hours of NH_4Cl acidosis *without any de monstrable* increase in renal glutaminase activity The enzyme activity does increase after more prolonged treatment but the capacity to excrete ammonia is not much increased over what it was in the twenty four hour acidotic animal In this same vein it should also be mentioned that in the dog all attempts by Rector and Orloff[36] to show an increase in renal glutaminase during pro longed acidosis have failed Therefore, in the capacity to increase ammonia excretion without an enzyme adaptation other factors in the biochemical reactions of glutamine metabolism must be operative

One such factor could be the changing rate of citrate and α ketoglutarate production with the reduced pH inside the tu bular cell in acidosis A smaller available supply of α ketoglu tarate could conceivably alter the equilibrium in glutamine me tabolism and favor free ammonia secretion into the urine More will be said about this idea in a larger context later in this chapter At the moment such an hypothesis remains purely speculative in the present discussion

At the time of this writing a paper has appeared by Madison and Seldin[37] purporting to show an adaptive increase in the hu man kidney during chronic acidosis in 'glycine oxidase, gluta minase asparaginase L-amino acid oxidase and D amino acid oxidase This work is open to such serious criticism that it de serves comment in the present discussion of enzymatic adaptation in renal ammonia production

Normal male subjects were made chronically acidotic by the ingestion of ammonium chloride at three increasing dose levels After measuring basal ammonia excretion at each level several amino acids were given by mouth and the augmentation in am monia excretion after each individual amino acid was measured The amino acids that had no augmenting effect were L-lysine and presumably other diamino acids Glutamic and aspartic acids

and proline caused a small but constant increment Glycine, glutamine, asparagine, L and D alanine, and L leucine each progressively augmented ammonia excretion in stepwise fashion paralleling the increase in NH_4Cl load

The authors conclude from these experiments that In the light of these considerations, the data indicate that glycine oxidase, glutaminase, asparaginase, L amino acid oxidase, and D amino acid oxidase show adaptive increases in the human kidney following the chronic administration of strong acids

These conclusions seem very risky and far reaching on the basis of the experiments and their design The amino acids were given by mouth, thus it is hard to know how their varying rates of absorption in the intestine and their metabolic assimilation in the liver altered them before they reached the kidney For this reason it seems very risky, particularly in the absence of any direct measurements of blood amino acids or kidney enzymes, to conclude that the particular amino acid given orally actually itself reached the kidney in sufficient concentration to 'adapt its particular enzyme The likelihood that the amino acid did not reach the kidney as such is strengthened by the work of Duda and Handler, discussed earlier, showing that amino acids passing through the liver are extensively converted to glutamine either directly or indirectly through transamination reactions Finally the paper overlooks the fact, discussed here, that such changes in ammonia excretion as those seen after amino acid administration can occur without any observable change in glutaminase

Before we will have a really clear picture of the biochemical mechanisms involved in renal ammonia production and its adaptive changes there will have to be clean cut experiments with isotopically labelled amino acids and their amides in which precursor product studies like those of Duda and Handler are performed on renal tissue, urine, and renal arteriovenous blood samples As yet the problem is not clear and with the exception of the apparently clear-cut results in the rat and guinea pig the problem of induced enzyme adaptation in ammonia excretion remains something of an enigma

tion must be one factor at least in the continuing capacity of the kidney to excrete ammonia However, it apparently cannot be the whole story Leonard and Orloff[25] have clearly shown in the rat that there can be a fourfold increase in ammonia excretion after only twenty four hours of NH_4Cl acidosis without any demonstrable increase in renal glutaminase activity The enzyme activity does increase after more prolonged treatment, but the capacity to excrete ammonia is not much increased over what it was in the twenty four hour acidotic animal In this same vein it should also be mentioned that in the dog all attempts by Rector and Orloff[36] to show an increase in renal glutaminase during prolonged acidosis have failed Therefore, in the capacity to increase ammonia excretion without an enzyme adaptation other factors in the biochemical reactions of glutamine metabolism must be operative

One such factor could be the changing rate of citrate and α ketoglutarate production with the reduced pH inside the tubular cell in acidosis A smaller available supply of α ketoglutarate could conceivably alter the equilibrium in glutamine metabolism and favor free ammonia secretion into the urine More will be said about this idea in a larger context later in this chapter At the moment such an hypothesis remains purely speculative in the present discussion

At the time of this writing a paper has appeared by Madison and Seldin[37] purporting to show an adaptive increase in the human kidney during chronic acidosis in glycine oxidase, glutaminase, asparaginase L-amino acid oxidase, and D-amino acid oxidase This work is open to such serious criticism that it deserves comment in the present discussion of enzymatic adaptation in renal ammonia production

Normal male subjects were made chronically acidotic by the ingestion of ammonium chloride at three increasing dose levels. After measuring basal ammonia excretion at each level several amino acids were given by mouth and the augmentation in ammonia excretion after each individual amino acid was measured The amino acids that had no augmenting effect were L-lysine and presumably other diamino acids Glutamic and aspartic acids

and proline caused a small but constant increment Glycine glutamine asparagine L and D alanine and L-leucine each progressively augmented ammonia excretion in stepwise fashion paralleling the increase in NH_4Cl load

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Site of urinary ammonia secretion and its relation to glutaminase distribution—It was first shown directly by Walker[38] in the amphibian nephron that glomerular filtrate proximal tubular and first part of distal tubular urine are ammonia free (Figure 35) Ammonia starts to appear in the middle of the distal

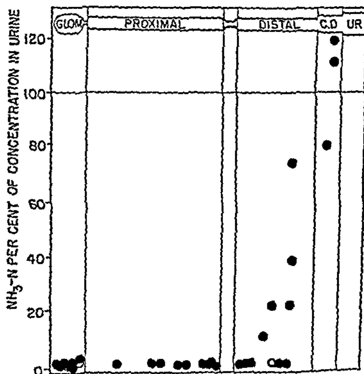


Figure 35 Observations from micropuncture studies on the nephron of the necturus showing the appearance of ammonia in the urine of the distal tubule and collecting ducts From Walker (38)

tubule and increases to a peak in the collecting duct Recent studies using the stop flow technique have confirmed the distal tubular site of ammonia secretion[39] That acidification of the urine also takes place in the distal segment further emphasizes the interrelatedness of this process and ammonia secretion as discussed in the first part of this treatise

Two recent studies[40,41] have been concerned with the distribution of the glutaminase enzymes in the various zones of the kidney of several species In dog and rabbit the kidneys were divided into cortex outer and inner medulla in rat and guinea pig into cortex plus another zone that included both outer and inner medulla In these several tissue zones assays were carried out for the three glutamine assimilating enzymes In all four species enzyme activity was highest in cortex and inner medulla in all species *glutaminase I* activity was always higher than either *glutaminase II* or *glutamine synthetase* The activity of these latter two enzymes was found only in outer medulla and cortex These studies show that the distribution of *glutaminase I* (the main glutamine hydrolyzing enzyme in ammonia synthesis?) is distributed in cortex where distal tubules are located and in inner medulla where collecting ducts converge toward the papilla Thus the distribution of *glutaminase I* correlates most closely with the nephron sites of ammonia secretion seen in Figure 35 Furthermore the presence of *glutaminase I* (and none of the other enzymes) in the area of the collecting ducts suggests the importance of the collecting ducts as a site of ammonia secretion

The Adrenals and Ammonia Excretion

It was reported by Jimenez Diaz[42] in 1936 that patients with Addison s disease show a decreased capacity to excrete ammonia Russell and Wilhelmi[43] subsequently observed that kidney slices from adrenalectomized rats show a similar decreased capacity to make ammonia *in vitro* A number of studies since then have attempted to learn whether these observations can be repeated under controlled experimental conditions and if so whether the mechanism of adrenal control of ammonia excretion can be explained on a mechanistic or teleologic basis

Harris Hartmann Rolf and White[44] observed that the adrenalectomized dog in whom water and salt output are maintained unaffected shows a similarly decreased capacity to excrete ammonia both in spontaneous and experimentally induced acidosis Desoxycorticosterone acetate administration repairs this defect Sartorius Calhoun and Pitts[45] subsequently presented similar conclusions based on experiments with rats

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In both these latter studies there were uncontrolled variables that require them to be interpreted with caution In those by Harris *et al*[44] there were no measurements of renal blood flow (RBF) or glomerular filtration rate (GFR) In their experiments where a defect of ammonia excretion was shown with so-called normal electrolyte and water excretion, the absence of these measurements means that the impaired ammonia excretion could have been related to renal hemodynamic collapse This same criticism must be applied to the experiments on the rat[45] whose RBF and GFR have been shown by Lipman[46] to be extremely labile during any kind of manipulation or stress The author has seen in his own laboratory that the administration of NH_4Cl to the adrenalectomized rat is often a shocking procedure and can reduce GFR by 50 per cent or more

It may be, as Schwartz Jenson and Relman[47] have reasoned, that sodium reabsorption itself is a stimulus to urinary acidification and ammonia excretion In their subjects sodium sulfate infusion produced a drop in urine pH and rise in rate of ammonia excretion only when sodium intake was low and the adrenal steroid compound F, was administered Leonard and Orloff[5] found exactly similar requirements for a sodium sulfate stimulation of urinary acidification in the rat *If this view is correct*, namely that acidification and ammonia excretion depend primarily on sodium retention, then the adrenal may play its role *in ammonia secretion* purely by facilitating this sodium reabsorption without any necessary change in general acid base balance of the body

Such an explanation has the added advantage that it removes the necessity of postulating an adrenal effect on substrate or enzyme aspects of ammonia production It is in harmony in this respect with the observations of White and Rolf[48] who found renal *glutaminase activity in the kidney of rat, rabbit, and dog* the same in normal and adrenalectomized states, and with the report of Wilson and Seldin[49] that the glutaminase adaptation occurs just as readily in the adrenalectomized as in the intact animal

Summary

The experimental evidence at hand seems to point to glutamine as the main direct source of urinary ammonia Amino acids appear to act in this capacity only in so far as they are converted to glutamine after deamination or transamination Glutaminase I the hydrolyzing enzyme present in both cortex and inner medulla stands as the most likely rate limiting enzyme system in the biochemical reactions leading to ammonia production The relation of the tricarboxylic acid cycle and the transamination deamidation systems to the reactions producing ammonia appears close and capable theoretically of altering the rate of the process α ketoglutarate a major reactant of both the tricarboxylic acid cycle and transamination deamidation occupies a central position in the complex of metabolic pathways crisscrossing glutamine assimilation Thus further work is needed on the relation of this metabolite to urinary ammonia production

Ammonia appears to diffuse as the free base (NH_3) along a pH gradient into acid urine where it is converted into the non diffusible ammonium ion (NH_4^+) by combining with H^+ ion concomitantly produced and secreted by the tubule cells The interrelation between this conversion hydrogen ion secretion and the conservation of fixed cation has been discussed

The distribution of glutaminase I correlates best with the demonstrated sites of ammonia secretion in the nephron of both the amphibian and mammalian kidney The adaptive increase in the capacity to excrete ammonia in chronic acidosis correlates well in certain species with the adaptive increase in renal glutaminase activity particularly glutaminase I In acute acidosis however increased ammonia excretion does not always correlate with an enzyme change and in the dog no adaptation in glutaminase even in chronic acidosis can be demonstrated Thus other factors in ammonia production must account for the change in ammonia excretion in these instances Although much attention has been given to the glutaminase problem and its changes in some species are most convincingly correlated with the capacity to excrete ammonia we must maintain an open mind to the possibility that the glutaminase changes where they are seen are experimental artifacts unrelated to the basic mechanism of adaptation

The meaning of the rise in ammonia excretion in acidosis is *teleologically understandable* However this is not immediately the case in the elevated ammonia excretion seen in alkaline urines in certain species The diffusion of ammonia into such highly alkaline urines runs counter to the mechanism of diffusion of ammonia and other bases which require an acid urine to produce a diffusion gradient and an ammonia trap Therefore it is tempting to ascribe the elevated ammonia excretion in alkalosis to the high rate of urine flow prevailing in those instances where it has been demonstrated That urine flow becomes such a limiting factor in ammonia excretion in alkaline urines has been clearly shown

The evidence from studies on adrenalectomized animals appears to the writer inadequate at present to support the firm conclusion that the capacity to secrete ammonia is reduced in adrenal insufficiency Renal blood flow is a labile function in the adrenalectomized animal and the acidotic stimulus is often a sufficient stress to reduce blood flow to near shock levels This in itself will reduce ammonia excretion Until this hemodynamic variable is carefully controlled it will be hard to assess accurately the capacity to excrete ammonia in the absence of cortical steroids

A possible role of the adrenal in ammonia excretion seems better derived from studies of intact rats and man relating acid and ammonia excretion to sodium retention without any change in general acid base balance Here the cortical steroids by promoting sodium reabsorption appear to stimulate ammonia excretion These studies perhaps do not take fully enough into account possible effects of the adrenal steroids other than their sodium retaining function The associated loss of potassium in the sodium restricted compound F treated men of Schwartz *et al*[47] might be expected to produce an intracellular acidosis which in turn could alter the susceptibility of the tubule cells to the acidifying effects of the sodium sulfate Nevertheless the exact role of the adrenal in ammonia excretion remains to be described fully

Relation between organic acid excretion in alkalosis and ammonia excretion in acidosis—In the last chapter we saw that citric and α ketoglutaric acids of the tricarboxylic acid cycle are

excreted at increased rates during metabolic alkalosis of the tubular cell interior It seems worthwhile at this point in a summary to relate this phenomenon to the problem of ammonia excretion

It has been suggested by Cooke and associates (Chapter IV) that the tubular secretion of organic acid anions may represent an exchange mechanism conserving fixed anion (chloride) in alkalosis in a manner analogous to the fixed cation conservation in acidosis through NH_3 and H^+ secretion Although the tubular secretion of citrate or α ketoglutarate remains to be demonstrated this anion exchange hypothesis remains an interesting idea worthy of investigation

In the light of this idea, it is tempting to postulate that the ammonia excretion of acidosis and the organic acid excretion of alkalosis may be two closely related phenomena, that is, may represent two different equilibrium states in a common biochemical mechanism We have seen that glutamine exists in a dynamic steady state with the tricarboxylic acid cycle through the reactions of transamination deamidation Now, in metabolic acidosis the evidence indicates that intracellular synthesis of citrate and α ketoglutarate is low Under these circumstances the excretion of these acids is almost nil while that of ammonia is high One could, therefore, visualize that in acidosis glutamine assimilation in the presence of a reduced supply of α ketoglutarate, shifts away from reductive amination toward an equilibrium favoring deamidation with diffusion of free ammonia into the urine

In contrast to this state of affairs, the shift toward alkalosis would produce a different situation The reactions in the cell would seem to lead in a different direction favoring organic acid production and reductive amination At the higher intracellular pH the condensing enzyme system would make more citrate which in turn would provide more α ketoglutarate to act as an ammonia acceptor in a reductive amination that would produce glutamate In alkalosis intracellular pH would be higher and thus closer to the pH optimum of the glutamine *transaminase deamidase system* of Meister *Under these circumstances* ammonia would be formed for the reductive amination of α ketoglutarate Ammonia would not diffuse into the urine, but organic acids being produced at a high rate would If this theory were

true one should find increased levels of glutamic acid in the renal tissue in intracellular alkalosis as a result of the postulated elevation in reductive amination To the writer s knowledge this observation has not been made but would constitute a crucial test of the theory

This concept would not only link ammonia and organic acid excretion as cation and anion conservation mechanisms but would also explain the mechanism of the dynamic shift from ammonia excretion in acidosis to organic acid excretion in alkalosis on the basis of a shifting metabolic equilibrium in the complex of bio chemical reactions involving glutamine the amino acids and the oxidative tricarboxylic acid cycle If this formulation were to be proven true on the basis of experiment it would explain how changes in intracellular pH or potassium concentration could affect a basic biochemical equilibrium and thus set into motion either cation or anion conservation mechanisms according to the needs of acid base balance at the moment

A comprehensive examination of the mechanism of ammonia excretion involves one in fundamental biochemistry of amino acids and their amides problems of cellular permeability and dif fusion enzyme adaptation and shifting steady states in complex reactions in response to changes in acid base balance at the whole body and cellular level It is necessary to see these basic mech anisms in a teleological light and to explain them in terms of the total economy of the body as it reacts to environmental change

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VI

ORGANIC ACIDS AND BASES

IN OUR DISCUSSION of metabolism and transport up till now we have considered for the most part metabolites synthesized in the body And in this context we have examined the tubular secretion of certain endogenous substances such as organic acids, like citric and malic and the weak organic base, ammonia In the present discussion we will consider the mode of excretion of certain organic acids and bases of *exogenous* origin substances which are for the most part foreign to the body These are stronger acids and bases, only slightly dissociated at pH of cell or tubular urine thus unlike the weak base, ammonia, they are relatively insoluble in lipids and therefore do not diffuse readily into or out of cells These compounds are filtered by the glomeruli and actively excreted by the tubules

The tubular excretion of phenol red and para aminohippuric acid (PAH) will receive major emphasis in the first part of our discussion These organic acid anions are of particular interest for several reasons within the context of renal physiology The mechanism of their tubular excretion is widespread among the animal species and, in addition, of the several foreign acids excreted by the tubules their mechanisms of transport have been the most carefully analyzed Thus a discussion of the tubular excretion of these and related substances must occupy a central position in any contemporary discussion of the metabolic aspects of renal function

The tubular excretion of organic bases involves mechanisms *related, but in some ways separate from that which handles these* exogenous organic acids Prototypes in this system may be represented by the sympatholytic drug tetraethylammonium chloride (TEA), and the metabolite of nicotinic acid, N methylnicotina

mide (NMN) The study of the mode of excretion of these two quaternary ammonium bases has presented us with interesting new problems in tubular transport and cellular metabolism Some of these will be considered in our discussion of weak organic base excretion in the second part of this essay

Phenol Red and Para Aminohippuric Acid

The first positive demonstration of renal tubular excretion of a foreign substance appeared in 1923 when Marshall and Vickers[1] showed that the dye phenol red is excreted by the tubules of the dog kidney The subsequent studies of Chambers and Kempton[2] emphasized the active nature of this mechanism *Using cultured cysts of chick embryo mesonephric tubules these workers observed the intraluminal accumulation of phenol red* in higher concentration than in the surrounding medium Within the next few years Shannon s[3] work on the excretion of phenol red in the dog appeared and then similar studies on man [4,5] These experiments established the fact that in both man and dog the clearance of phenol red at low plasma levels is at or near the rate of renal plasma flow They further showed that phenol red is excreted by glomerular filtration and tubular excretion this latter process exhibiting a maximal rate (Tm) at elevated plasma levels

It is now a matter of history that a large number of other weak organic acids are also excreted by the tubules and that *these substances compete with one another for what appears to* be a common transport mechanism Some of these are Diodrast hippuran p aminohippuric acid p aminophenaceturic acid p acetylaminohippuric cinnamoylglycine penicillin and phlorizin

The introduction of the PAH clearance as a measure of effective renal plasma flow[6] brought this member of the series into special prominence In the studies that have followed on the nature of the transport mechanism of these organic acids the bulk of experimental work has centered on phenol red (and other dyes) and PAH (and related derivatives of benzoic and hippuric acid) Therefore it is the studies with these two classes of compounds which must be followed to see what has been learned about exogenous organic acid transport in the kidney

Höber[7] has extensively analyzed the physico-chemical aspects of the active tubular transport of a number of acid dyestuffs in the frog kidney Using sulfonic azodyes and a series of naphthalene and benzene derivatives, he developed a 'nonpolar polar theory to explain the molecular basis for transport of the dye He assumed that the transfer of a nonpolar polar dye depends on a preliminary orientation and attachment of the molecules to the acting organic structure probably to the cell surface, by the organophilic half, while, by a suitable partial rotation of the elongated molecules the hydrophilic half could be anchored in the aqueous surroundings He conceived this loose attachment by van der Walls forces to precede the actual active transfer process The more intimate nature of the bonding between dye and surface site was not discussed by Höber however, his studies were classic in the way they related critical molecular structure to active transfer, and they have had a great influence on subsequent approaches to this difficult problem One such study will be discussed later in an attempt to relate transfer capacity to molecular structure in a group of benzoic acid derivatives

Since these earlier studies with phenol red excretion and the physico-chemical aspects of dye transport, this field has received valuable attention from Forster and his colleagues With the introduction of the teased tubule preparation of the flounder and the thin slice of kidney cortex,[8] a valuable tool was provided for studying certain aspects of transport *in vitro*

Using the flounder tubule Forster and Taggart[9] studied certain metabolic aspects of phenol red transport from suspending medium into the tubule lumen Under the conditions of their experiments this process requires oxygen, is depressed by cold and a variety of metabolic inhibitors including certain heavy metals, iodoacetic acid, cyanide azide, and 2,4-dinitrophenol In an extension of this work with 2 4-dinitrophenol, Taggart and Forster[10] tried a number of different substituted phenols and found a good correlation between capacity to 'uncouple" oxidation and phosphorylation (as evidenced by stimulation of respiration in kidney mince) and capacity to block phenol red transport Picric acid was the only exception to the correlation It is not a

typical uncoupler its inhibitory effect is believed by the authors to be non specific

These data with 2,4 dinitrophenol, and other substituted phenols, present rather convincing evidence that phenol red trans port requires intact coupling between oxidation and phosphoryla tion It is unlikely that the phenol red itself is phosphorylated during transport Since the concentrative transfer of phenol red in the cell or tubule lumen requires the expenditure of energy, it is more likely that the requirement for oxidative phosphoryla tion relates to the elevation of the carrier to a higher energy level at a certain place within the cell or its membranes

The studies with nitrophenols have a more specific meaning in terms of the transport mechanism than do the experiments with the less specific inhibitors such as mercury, cyanide, azide, iodoacetate, etc It is difficult to interpret effects of these inhibitors in terms of any one enzyme system as it functions in the overall transport mechanism The good correlation between transport inhibition and stimulation of respiration in the case of the un couplers affords a basis for a more specific interpretation in terms of site affected than do studies with iodoacetate or heavy metals These may affect a number of enzyme sites in a non specific way with or without general effects on respiration The same may be said for azide and cyanide which are known to affect potassium transport under circumstances where oxygen consumption re mains unaffected [11]

Steps in the transport process—The overall process of trans porting a substance from peritubular fluid into the tubular lumen involves at least two barriers or steps *Step I* represents trans port across the cell membrane on the peritubular side of the cell with subsequent intracellular accumulation *Step II* is the trans port across the luminal membrane into the tubular fluid

The tubular excretion of a series of dyes and PAH has been analyzed both *in vivo* and *in vitro* in an attempt to understand the role of these two separate steps in the transfer process No matter which species was examined, the overall process shows dependence on cellular respiration with coupled phosphorylation In addition there are competitive interrelations among the several substances transported

In isolated surviving tubules of flounder[12 13] or other cold blooded forms several dyes of phenolsulfonphthalein series are actively transported from suspending medium into tubule lumen *in vitro* This occurs under fixed conditions which favor aerobic metabolism The process occurs at room temperature with buffered balanced saline medium These dyes accumulate within the tubular lumen in concentration higher than in the medium thus active transport by definition has occurred Phenol red and chlorphenol red are most active in this regard Certain other dyes are sluggishly taken up but rather than appearing in the lumen they accumulate within the cell as Hong and Forster[13] say they are trapped in the cell These trapped dyes block the intraluminal accumulation of those like chlorphenol red that are more actively transported Similarily cold anoxia azide cyanide and 2 4-dinitrophenol inhibit the uptake of the active dyes Since both these inhibitors and the trapped dyes prevent the initial uptake of chlorphenol red or phenol red from the medium Hong and Forster[13] conclude that the transport across the peritubular cell membrane (Step I) is metabolically linked and subject to competitive inhibition by the several dyes in the series

Using the isolated flounder tubule preparation Puck Wasserman and Fishman[12] examined these two steps in a different way They found that Step I (from extracellular fluid to inside of the cell) in the flounder is active dependent on coupled oxidation and phosphorylation and specifically requires potassium ion Step II (from cell into tubule lumen) also displays a specific but different ionic requirement in this case for calcium In its absence for instance phenol red will accumulate in the cell but not in the lumen If both calcium and potassium are missing phenol red enters neither cell nor lumen Thus the active transfer of phenol red in the flounder tubule involves both steps each of which is active and requires energy

In a more recent study Hong and Forster[14] have analyzed the role of Step II in dye transport in another way In their experiments flounder tubules were allowed to accumulate chlorphenol red or phenol red until the dye was in high concentration in the lumen The tubules were then transferred to an ice box or kept at room temperature and observed for run out of the

dye from the lumen It was found that cold as well as competi tive or metabolic inhibitors facilitated the run out of the dye a process interpreted by the investigators to mean an inhibition of Step II They visualized the ability to maintain high intraluminal dye concentration as a manifestation of the continued *active* trans port of dye across the luminal cell membrane if this process is blocked by cold anoxia or inhibitors the dye diffuses back through the cell into the suspending medium This is called run out The parallel but inverse relation between conditions affecting both uptake and run out of dyes in the cold water flounder tubule seems to indicate as the authors concluded that transport across the membranes on either side of the cell is active and displays specific ionic and energy requirements

These two steps of the transport process show important similarities as well as differences in the warm as compared to the cold blooded kidney Using thin slices of kidney cortex Forster and Copenhaver[15] have studied the accumulation of PAH and a number of dyes in the rabbit tubule In this system the slice is able to accumulate phenol red chlorphenol red or PAH so that the concentration of substance in the slice is some 10 to 20 times higher than in the medium This concentrative uptake is ex pressed in terms of the slice to medium ratio (S/M)

Microscopic examination of these rabbit kidney slices showed that none of the dye accumulates in the tubule lumen as it does in the flounder frog turtle dogfish and chick mesonephric cyst It is concentrated only within the cell Metabolic and competi tive inhibitors including Diodrast and Benemid affect this intra cellular accumulation in a manner entirely analogous to the intraluminal accumulation process seen in the cold blooded species It seems logical to conclude therefore that active trans port of dyes and PAH in the mammalian tubule cell involves only *one step rather than two namely transport across the peritubular* cell membrane with intracellular accumulation The active trans port step from inside the cell across the luminal membrane is missing in these warm blooded tubules

The implications of this difference raise several interesting questions Forster[16] has suggested that it may be related to the differing rates of glomerular filtration in the kidneys of the two

types of animals The warm blooded mammal with a high rate of glomerular filtration maintains a rapid flow of fluid down the proximal tubule This fluid flowing rapidly past the tubular cell that is loaded with organic acid constitutes a fluid phase of es sentially zero organic acid concentration and thus a diffusion gradient between cell and tubular fluid is created What is more, the continued renewal of this fluid phase as a result of continued glomerular filtration maintains the diffusion gradient and thus in these species no active Step II is required at the luminal border diffusion is adequate to facilitate movement out of the cell

On the contrary however, in those cold blooded species with a low rate of glomerular filtration such a self perpetuating diffusion gradient between cell and tubular urine does not exist With the sluggish urine flow of these species the tubular fluid constitutes essentially a static extracellular fluid compartment Under these circumstances intracellular dye or PAH would simply come into diffusion equilibrium with this static fluid phase and very little movement out of the cell would take place However, with the existence of an active transport at the luminal border of the cell a concentrative transfer of dye can take place as it is actively lifted to a level of higher concentration in the tubular fluid Thus the cold water animal with a very low rate of glomerular flow is able to excrete substances by active two-step excretion in the absence of the ready made diffusion gradient afforded the warm blooded animal by virtue of his high rate of glomerular filtration

This theory has recently received some experimental verifica tion in studies on rabbits by Foulkes and Miller [17] Urinary con centration of PAH during mannitol diuresis was taken to repre sent essentially that concentration of PAH obtaining in the proxi mal tubules where its excretion occurs Since urine PAH con centration under these circumstances was always substantially below that found in renal cortical tissue from rabbits excreting PAH at Tm rates, it was concluded that a concentrating step at the luminal border of the cell was not required for PAH excre tion into the tubular urine These findings were strengthened by the additional observation that stop flow fractions of urines containing PAH showed essentially the same PAH concentrations

(after correction for water reabsorption) as did bladder urines during mannitol diuresis

Thus these experiments are in harmony with the rabbit kidney cortex slice studies of Forster and Copenhaver Proximal tubular urine PAH concentration is apparently enough below intracellular PAH levels even under saturation conditions, that an active transport step at the luminal border is not required It is hoped that similar studies, perhaps with micropuncture techniques, can be done on cold blooded species

Nature of the transport mechanism for PAH—Stimulated by the studies of Stern, Eggleston Hems, and Krebs[18] on the concentrative transport of glutamate in brain slices and the work of Chambers and Kempton on phenol red transport in the chick mesonephros Cross and Taggart[19] in 1950 introduced an adaptation of Forster s technique[8] for studying active uptake of dyes in thin slices of kidney cortex and used this preparation in an important study of PAH transport *in vitro* They showed that slices of rabbit kidney cortex are capable of accumulating PAH against a considerable concentration gradient in a buffered balanced saline medium This process is dependent on oxygen and is considerably more rapid at 37° than at 25° C It is depressed by a variety of inhibitors, among them 2,4 dinitrophenol, in uncoupling concentrations where oxygen uptake definitely is elevated Thus the transport of PAH *in vitro* resembles in many ways that seen for certain dyes in the flounder tubule

Perhaps of greatest subsequent interest in this work was the observation that the uptake of PAH in the rabbit cortex slice was stimulated by acetate and to a lesser extent by pyruvate (Fig 36) The uptake was found to be inhibited by dicarboxylic acids of the citric acid cycle and a number of fatty acids of intermediate chain length (C_5 C_{10}) In general the longer the fatty acid chain length the greater the inhibition These observations of acetate stimulation and fatty acid inhibition were to play a major role in the subsequent investigation of the PAH transport mechanism and proved the jumping off point for an intensive investigation by Taggart s group and a number of other workers

In an ingenious study in the intact dog, Mudge and Taggart[20,21] were able to show that many of the same conditions that affect PAH accumulation in the kidney cortex slice likewise affect PAH excretion in the living kidney Thus acetate stimulates and 2,4-dinitrophenol depresses Tm PAH in the dog This latter effect on Tm PAH occurs without any change in the reabsorptive Tm for glucose Again, as in the slice, succinate and fumarate as well as certain other metabolic intermediates depressed the Tm for PAH An experiment of Mudge and Taggart showing the stimulating effect of acetate on Tm PAH is shown

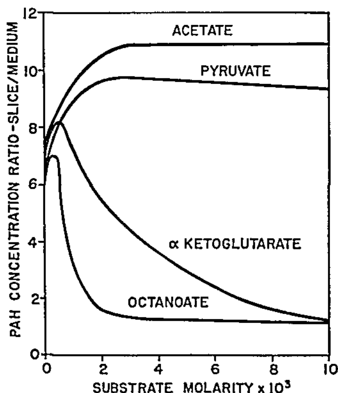


Figure 56 The effect of acetate pyruvate α ketoglutarate and octanoate on the uptake of PAH by thin slices of kidney cortex *in vitro* For discussion, see text. From Cross and Taggart (19)

in Table V These effects of acetate and 2,4-dinitrophenol on PAH transport both *in vitro* and *in vivo* led to an interesting line of speculation and experiment on the nature of the carrier for PAH in the kidney

TABLE V

EFFECT OF ACETATE ON $T_{M_{PAH}}$

Time	C_r	V	p Aminohippurate				Tm
			P	UV	$C_r \times P$	Tm	Average Control
min	ml/min		mg %		mg/min		%
− 41	4 gm creatinine and 4 gm PAH in 100 ml water i v						
− 37	Sustaining infusion started 7.5 mg creatinine and 27 mg PAH per ml in isotonic saline at 2 ml/min						
0–20	62.8	2.40	61.4	42.8	38.6	10.2	92
20–40	70.2	3.95	59.1	52.8	41.4	11.4	103
40–60	66.3	5.42	58.3	50.3	38.7	11.6	105
60	Second infusion started 0.8 mg PAH per ml in Na acetate 280 mEq/l at 15 ml/min						
60–80	73.8	10.3	58.0	60.2	42.8	17.4	158
80–100	75.4	13.3	56.2	62.2	42.4	19.8	180
100–120	69.2	15.5	55.4	58.9	38.3	20.6	187

The experiments with 2,4 dinitrophenol clearly showed that a supply of high energy phosphate is required This observation and the acetate stimulation were fitted into a working hypothesis along these lines Beyer and his associates had made the interesting observation that Benemid, which inhibits PAH transport *in vitro* and *in vivo,* likewise inhibits the synthesis of PAH from p aminobenzoic acid (PAB) and glycine in kidney Concentrations of Benemid that accomplish this inhibition do not interfere with either oxygen consumption or oxidative phosphorylation Both Beyer[22,23] and Taggart[24] reasoned that the transport of PAH might involve its hydrolysis to PAB and glycine and then the resynthesis of PAH from these compounds

Cohen and McGilvery[25] had studied the synthesis of PAH from PAB and glycine and had shown that the reaction requires high energy phosphate It seemed reasonable, therefore, that the

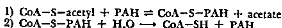
effects of 2 4 dinitrophenol and Benemid on PAH transport could be explained by assuming that both these agents interferred with the PAH resynthesis step in the hypothetical transport scheme

This idea however was effectively ruled out by an experiment of Taggart s[24] with PAH labelled with C^{14} in the carboxyl group The assumed hydrolysis and resynthesis of PAH would result in the dilution of the isotopic carboxyl carbon by the incorporation of unlabelled glycine carbon in the PAH during the resynthesis phase The administration of the C^{14} PAH to a dog and the recovery of the PAH from the urine showed that the specific radio-activity of the recovered and original PAH were identical hence it was very unlikely that the postulated scheme of hydrolysis and resynthes s was valid

Taggart then turned his attention to the possible role of coenzyme A in PAH transport scheme[24] To invoke coenzyme A seemed logical for several reasons not the least of which was that it explained the apparent unique role of acetate in PAH transport Lynen Reichert and Rueff[18] had clearly shown that the active form of the acetate that is condensed in the tricarboxylic acid cycle is the acetyl mercaptan of coenzyme A (CH_3CO S CoA) In the formation of this compound ATP is required The likelihood that acetyl CoA was involved in PAH transport loomed large in light of the observation by Beyer s group[22] that Benemid inhibited both PAH transport and hippurate synthesis as discussed above Chantrenne[7] observed that coenzyme A is required for hippurate synthesis in Cohen and McGilvery s system and when Schachter and Taggart[28] subsequently prepared benzoyl S CoA as the activated form of benzoic acid in the hippurate synthetic system the coenzyme A theory gained strength

With these facts in front of them and with the knowledge that many other acyl mercaptans of CoA had been prepared such as butyryl CoA succinyl CoA palmityl CoA and then Benzoyl CoA Taggart and his group were led to speculate that PAH and various other carboxylic acids that are either transported or like Benemid block transport likewise form acyl mercaptans with CoA In such a scheme acetate would play a central role in an obligatory exchange reaction facilitating either the synthesis or

degradation of the hypothetical intermediate, p aminohippuryl CoA These reactions were visualized as follows



Thus PAH would be carried to a higher energy level in combination with the carrier as the coenzyme A form The simple hydrolytic cleavage of this complex would liberate PAH into the cell and allow it to accumulate there in higher concentration than outside The overall reaction would involve the conversion of ATP to AMP and pyrophosphate and thus would invoke ATP as the energy source for the active transport The scheme also would allow for the recycling of acetate and would thus explain the fact that it stimulates PAH transport in catalytic amounts

This theory, put forward by Taggart as frank speculation [24] was recently subjected to a critical examination in his own laboratory [29] and was shown by a decisive experiment to have serious limitations On the grounds that it is the carboxyl group of the PAH *that reacts with the carrier (perhaps to form the PAH-S CoA* derivative), PAH labelled with O^{18} in the carboxyl group was *administered to see whether such bonding could take place dur* ing PAH transport The labelled PAH was slowly infused into the renal artery of one kidney of a dog Of 82 mg so given, 72 mg were recovered from the ureter of the infused kidney, and it was shown that some two thirds of this entered the urine by tubular excretion Taggart postulated that information concern ing the extent of loss or retention of the carboxyl O^{18} in the PAH during tubular transport should set limits on the type of mech anism involved In particular it was evident that CoA bonding of the type outlined above would result in appreciable loss of car boxyl oxygen

When the labelled PAH isolated from the urine was degraded and analyzed for its O^{18}, it was shown quite conclusively that there had been *essentially no loss of carboxyl* O^{18} *This decisive* experi ment showed therefore, that the CoA bonding with the carboxyl group could not occur as Taggart had originally visualized it The experiment also ruled out an amide bonding between car boxyl and amine groups (CO NHR) [30]

Taggart has posed other possible types of bonding between carboxyl group and PAH[30] and besides ruling out thiol ester and amide bonding he also considers an ester type (CO O CH_2R) as unlikely because of the acyl-oxygen bond (CO O) is more liable to hydrolytic cleavage than is the methylene-oxygen bond (O CH_2) Cleavage of the CO O bond would entail loss of carboxyl oxygen More likely carboxyl bonds would be a car boxylic phosphoric anhydride (CO O PO_3HR) or an ionic type of bonding between PAH and carrier (COO^-R^+) Neither of these can be ruled out by the O^{18} experiment

Recently Kellerman[31] working in Taggart s laboratory has prepared benzoyl and hippuryl adenylates Kellerman has shown that enzymatic cleavage of both these adenylates takes place at the O P bond in the molecule Furthermore he has observed that it is possible to join a carboxyl group in covalent linkage of the type in the acyl adenylate and to liberate it again without loss of its original oxygen atoms either to the surrounding medium or to other reactants

These facts are interesting in light of Taggart s finding that O^{18} labelled PAH suffers no loss of its carboxyl oxygen during transport Although Kellerman is careful not to make undue claims for the acyl adenylates in carboxylic acid transport his beautiful work is consistent with the idea that the acyl adenylate of hippurate and PAH may be formed in the reactions with the carrier during their transport That benzoyl adenylate and hip puryl adenylate would be handled quite differently however will be seen below when we examine some recent experiments in molecular specificity of transport in this system

Although excluding the coenzyme A thiol ester theory of PAH transport the O^{18} and adenylate studies still leave unex plained the stimulation of PAH transport by lactate pyruvate and acetate its inhibition by low concentrations of the dicar boxylic acids of the cycle and by fatty acids of intermediate chain length (C_6 C_{10}) However recent experiments by Schachter Manis and Taggart[32] have thrown new light on the meaning of these phenomena

These workers have studied the synthesis degradation and active accumulation of a number of aliphatic acyl glycines by

kidney tissue of several species It has been shown that these synthetic reactions, like that for hippurate, involve the formation of the acyl thioesters of coenzyme A which are the energy rich intermediates in acyl glycine synthesis The reactions are as follows

1) $R-COOH + ATP + CoA-SH \rightleftharpoons R-CO-S-CoA + AMP + PP_i$
2) $R-COS-CoA + NH_2-CH_2-COOH \longrightarrow R-CO-NH-CH_2-COOH + CoA-SH$

Further studies showed that a series of these acyl glycines were themselves actively accumulated by kidney cortex slices However, since they are simultaneously hydrolyzed by enzymes in the kidney at rates decreasing with lengthening carbon chain of the acyl group, significant S/M ratio could only be observed with long chain acyl glycines such as n valeryl and isocapryl gly cine The important observation was then made (Figure 37) that

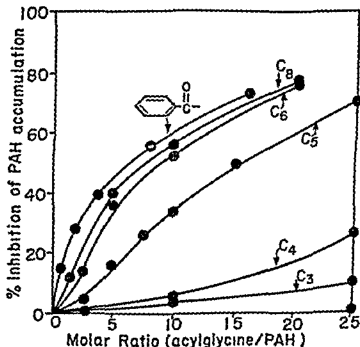


Figure 37 Experiments correlating the chain length of a number of aliphatic acyl glycines with their inhibition of PAH uptake by thin slices of kidney cortex *in vitro* The effect of hippuric acid is also shown From Schachter, Manis and Taggart (28)

the series of aliphatic acyl glycines (and the aromatic acyl glycine, hippurate) inhibit the active uptake of PAH by rabbit kidney cortex slices Acetyl glycine (C_2) was not at all inhibitory butyryl glycine (C_3) and propionyl glycine (C_4) were moderately so However, as the acyl chain length increased to 5, 6 and 8 carbons, marked inhibition of PAH uptake was apparent This observation provided a reasonable explanation of the inhibition of PAH transport *in vitro* and *in vivo* by fatty acids of intermediate chain length These fatty acids undergo conjugation with glycine, and the acyl glycine thus formed competes with PAH for transport

The stimulatory effect of acetate on PAH transport is explained as follows In the kidney there is a pool of endogenous fatty acids whose CoA derivatives become available for acyl glycine synthesis It is reasoned that a continuous endogenous tissue level of these longer chain acyl glycines which are hydrolyzed at a low rate, compete with PAH for transport and thus represent a continuous physiologic inhibition of the mechanism The administration of acetate floods the tissue with this anion which then usurps the available glycine to form acetylglycine This substance is not inhibitory to PAH transport (Figure 36) and thus 'releases the prevailing inhibition caused by the presence of the other acylglycines which are slowly hydrolyzed and disappear

This explanation is enhanced by the observation shown in Figure 38 that the acetate stimulation of PAH transport occurs only in those species (dog rabbit pigeon and guinea pig) whose kidneys are capable of forming acylglycines In those species that are unable to do this (chicken, turkey duck, goose and dogfish), acetate addition does not stimulate PAH transport For some unexplained reason it actually depresses PAH uptake in dogfish kidney (bottom curve Figure 38) The inhibitory effects of dicarboxylic acids of the citric acid cycle are unexplained by this acylglycine formulation

This fascinating trail of research although not yet offering a detailed description of the organic acid carrier (such as that worked out for amino acids in the ascites tumor cell), has explained certain of the inhibitory and stimulatory effects of carboxylic acids and hippurate Unexplained is the nature of the

carrier for phenol red, Diodrast, penicillin, phlorizin and other substances transported by the same system? Likewise the inhibition of PAH transport by Benemid is hard to understand either

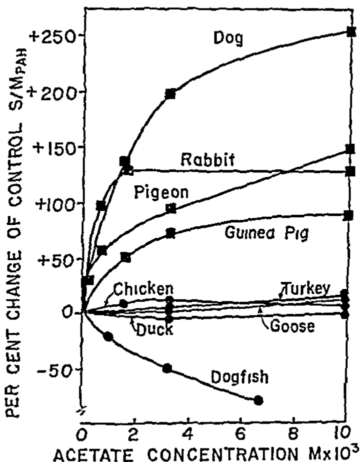


Figure 38 Effect of acetate concentration on PAH accumulation by thin slices of kidney cortex in various species Species capable of synthesizing acyl glycines (PAH) are designated by (■) species not possessing this activity by (●) From Schachter Manis and Taggart (28)

in terms of the appearance of competing acylglycines or the inhibition of hippurate synthesis If Benemid inhibition of hippurate synthesis were to do anything in terms of acylglycine competition in PAH transport, one would expect it to stimulate rather

than inhibit since in its presence there would be decrease in tis sue hippurate concentration and thus removal of a competitive inhibitor of PAH transport Thus Beyer s correlation[23] between carinamide and Benemid depression of PAB glycination and PAH and phenol red transport although impressive is unexplained in terms of the acyl glycine formulation

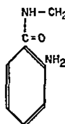
The relation between Kellerman s observations that benzoyl adenylates can be formed and Taggart s list of possible carboxyl bonds remains incomplete at present That PAH and the other carboxylic acids that share the tubular excretory system must be activated to effect their concentrative transfer into the renal cell is a thermodynamic necessity in terms of active transport That high energy phosphate should be implicated in the mecha nism and probably at this activation point seems logical and well established from the data at hand That the inhibition of organic acid transport by Benemid may be unrelated to either these energy reactions or the inhibition of PAB glycination is evi denced by the fact that it inhibits PAH transport in the absence of any inhibition of oxidation or phosphorylation [19] And further more it is hard to relate the Benemid effect on the transport of such disparate substances as PAH phenol red Diodrast and urea[13] to either its carboxylic acid structure or its inhibition of PAB glycination

Höber s nonpolar polar theory has pointed out the necessity for organophilic and hydrophilic groups on the transported mole cule His theory suggests an ionic bonding between acid and car rier on the membrane an idea also suggested by Taggart These thoughts bring us then once more to the central and recurring problems of molecular specificity in this transport system

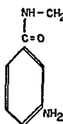
Molecular specificity for transport of benzoic and hippuric acid derivatives—Knoefel and Huang[34] and Despopoulos Archer Huang and Knoefel[35] have provided valuable information con cerning organic acid transport utilizing a series of substituted benzoic and hippuric acids Benzoic acid itself and a group of iodo- amino- or hydroxy substituted benzoic acids all show net reabsorption in the dog kidney However substitution of benzoic acid with acylamido groups (acetamido or even better propiona

mido) in the 2, the 4, the 3, or the 3,5 positions causes tubular excretion of the compound[34]

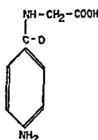
In a more detailed analysis of specificity of the acylamido structure, Despopoulos *et al*[35] have made parallel studies of the rabbit kidney cortex slice accumulation and the mode of renal excretion of a series of these substituted benzoic and hippuric



ortho
aminohippurate

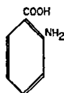


meta
aminohippurate



para
aminohippurate

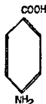
acids * All three of the following amino substitutions of hippuric acid are accumulated by the slice and excreted by the tubules In each of these the acylamido group is present and its proximity to the amino group does not seem to affect its capacity for trans port Nor is the amino group necessary since hippuric acid itself is excreted[36] None of the following amino substituted forms of benzoic acid is excreted by the tubules



ortho



meta



para

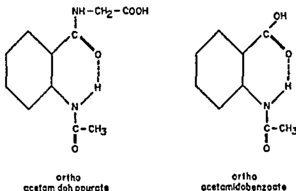
Benzoic Acid

However, if one changes the carboxyl group here to an aceta mido group in either meta or para substituted forms, these

*The formulae shown on this and the next page do not appear in the refer ence No 35 cited above However Dr Despopoulos has given permission for their inclusion here

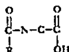
acetamido benzoic acids are avidly accumulated by the slice and excreted by the tubules

Neither ortho acetamidohippuric nor ortho acetaminobenzoic acids are accumulated or excreted Despopoulos believes that if the acetamido group is spatially oriented too close to another acylamido group (as in ortho acetamidohippurate) or a carboxyl group (as in ortho acetamidobenzoate) some sort of closure by intramolecular attraction occurs (as visualized by the dotted lines between hydrogen of the acetamido group and the oxygen of the other acylamido or carboxyl) and this prevents bonding of the compound with the transport carrier



These studies point to the importance of the acylamido group in imparting transport capacity to these benzoic acid derivatives

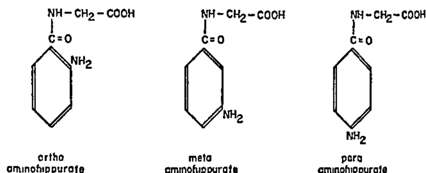
From more recent studies however, Despopoulos[37] has reached the conclusion that the acylamido hypothesis, although consistent in the series of compounds whose study supported it, may be too limited Working with an even larger series of compounds, aliphatic as well as aromatic, Despopoulos believes that the critical structure, whether in ring or straight chain organic acid must be as follows



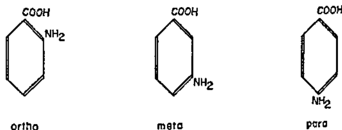
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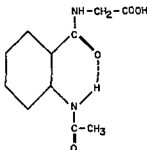
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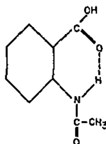
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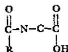
ortho acetamidohippurate



ortho acetamidobenzoate

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From more recent studies, however, Despopoulos[37] has reached the conclusion that the acylamido hypothesis, although consistent in the series of compounds whose study supported it, may be too limited Working with an even larger series of compounds, aliphatic as well as aromatic, Despopoulos believes that the critical structure, whether in ring or straight chain organic acid must be as follows



It so happened that in all their earlier compounds reported and discussed above "R" was a ring structure, but apparently it can

Tetraethyl-ammonium
Methyl-nicotinamide
Hippurate
m-Amino-hippurate
Diodrast
Iopax (Uroselectan)

Phenol-red
Penicillin
p Amino-hippurate
p-Acetylamino-hippurate
Neo-iopax
2-Pyridone-I-acetic acid

Ponceau *R*
Caronamide
Hippuran
o-Hydroxy-hippurate
Skiodan

Figure 39 Structure of molecules known to be excreted by the kidney tubule From Wilbrandt (51)

be straight chain as well It is the ketone group separate from the carboxyl by a nitrogen and a carbon that appears to represent the critical configuration

Such groups or ones closely related are seen in the molecules of many substances showing excretory transport in the kidney (Figure 39) This fact is apparent when one looks at their form ulae especially hippurate m aminohippurate Diodrast Iopax p-aminohippurate 2 pyridone 1 acetic acid Hippuran and O hydroxyhippurate

Tetraethylammonium and N methylnicotinamide are organic bases not sharing the organic acid mechanism they will be dis cussed separately below It is hard to see how phenol red fits the scheme of critical structure It fits Hőber s nonpolar polar theory but it has neither carboxyl nor nitrogen and it would stretch the imagination to try to see its structure in any way an alogous to the Despopoulos scheme Thus phenol red and many dyes whose transport shares many aspects of the mechanism for the benzoic and hippuric acids must remain in a separate bio chemorphological class at present It is hoped that further studies on their critical molecular structure will be carried out to extend the classic experiments of Hőber and allow us to go further in describing their transport mechanism

The key to the nature of the bonding between substrate and carrier for the substituted benzoic acids (of which PAH represents a classic type) may come from a combination of the approaches using various substituted benzoic acid molecules on the one hand and critical isotope distribution experiments such as Taggart s with O^{18} labelled PAH on the other Many fruitful avenues seem open for research in this direction One thinks back in this regard to the elegant biochemical analysis that drew heavily on known chemical reactions between chelated amino acids and the vitamin pyridoxal A similar approach with the acylamidoben zoates might prove fruitful Do they chelate with metals or hy drogen? The abolition of transport that occurs when one acyl amido group comes too close to a carboxyl or another acylamido oxygen in the molecule suggests the possibility of an oxygen hydrogen bond or a chelation between carboxyl group metal (in membrane) and nitrogen in the acylamido group The investiga

tion of metal chelates of acylamidobenzoic acids should be carried out and a comparison made between critical molecular structure, capacity to form chelates and transportability by kidney *in vitro* and *in vivo*

Thus we have seen a trail of research in active tubular excretion that started back with Marshall and Vickers and Chambers and Kempton, both groups showing in different ways the tubular excretion of phenol red The trail has led through a vexing yet brilliant series of studies on the various aspects of the process its steps, its comparative physiology and biochemistry, its molecular specificity, its dependence on metabolism and high energy phosphate, and its competitive interrelations with the several member molecules that ' use the mechanism And now we are at the point where we seem about to understand the nature of the *carrier and its association with the substrate during the transport process* It is this chapter in the story that awaits writing

Organic Bases

Like the organic acids which we have just discussed, the elimination of a number of organic bases by the kidney involves not only glomerular filtration but tubular excretion as well Sperber was the first to demonstrate the tubular excretion of the quaternary base, N methylnicotinamide (NMN), in the chicken [38] Beyer and his colleagues subsequently showed in 1950 that NMN is also handled in this way by the dog kidney [39] These same excretory characteristics were shown for tetraethylammonium (TEA) by Rennick and her colleagues in 1954[40] and for mepiperphenidol (Darstine) by Beyer and his associates in 1953 [41] Orloff, Aronow, and Berliner[42] showed in that same year that the non quaternary base, 2 benzyl 2 imidazoline (priscoline) is also excreted by the renal tubules

A body of impressive evidence supports the conclusion that these organic bases are not transported by the same mechanism that handles *the organic acids such as phenol red and PAH* The organic base mechanism is not inhibited by Benemid or PAH Neither in turn do any of the organic bases inhibit the tubular transport of PAH In addition, the two mechanisms are differentiated on the basis of their sensitivity to the basic cyanine dye #863

Rennick, Kandel and Peters[43] have recently shown that both TEA and NMN excretion by the tubules of the dog and chicken are markedly inhibited by this dye In the dog, for instance, as little as 1 25 μMol per kilogram of body weight blocks the transport of these bases This phenomenon for TEA and NMN is seen in the diagram of Figures 40 and 41 That PAH and phenol red transport are not inhibited by this cyanine dye further emphasizes the separateness of the organic acid from the organic base transport mechanism

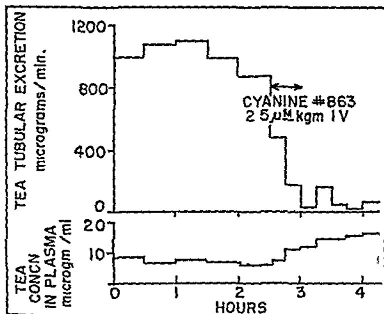


Figure 40 Inhibition of the tubular excretion of tetraethylammonium by the basic dye cyanine #863 in the dog From Rennick Kandel and Peters (43)

This cyanine dye #863 is highly concentrated in the kidney where it is avidly attached and not easily dissociated from the cells Differential centrifugation studies have shown that it is predominantly located in the mitochondria Since the mitochondria

house the enzymes of most of the reactions of oxidative phosphorylation in cells, it is tempting to speculate that cyanine dye may inhibit organic base transport by disrupting oxidative phosphorylation This speculation is strengthened by the fact that certain other dyes, such as methylene blue, are known to be ' uncouplers

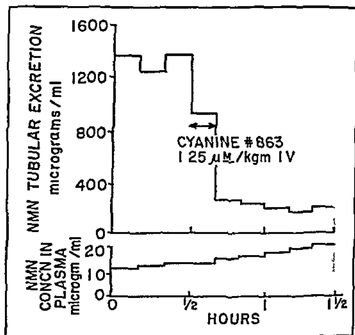


Figure 41 Inhibition of tubular excretion of N methylnicotinamide by the basic cyanine dye #863 in the dog From Rennick Kandel and Peters (43)

of oxidation and phosphorylation However, Rennick *et al*,[43] thinking of this possibility, point out that the concentrations of cyanine dye #863 required to inhibit oxidation and phosphorylation are much higher than the amounts that they found to be present in mitochondria Thus the dye inhibition of the base transport must be on some other basis, perhaps a competition with the base at a higher degree of affinity for some component of the carrier system

Like the organic acids the tubular excretion of certain of these organic bases reaches maximal rates at high loads This

fact in the case of TEA excretion in the dog is illustrated in Figure 42. These data are taken from the studies of Rennick, Calhoon, Gandia, and Moe.[40]

Recently several groups of investigators have attempted to understand organic base transport by use of the slice[19] with studies similar to those discussed above in connection with PAH.

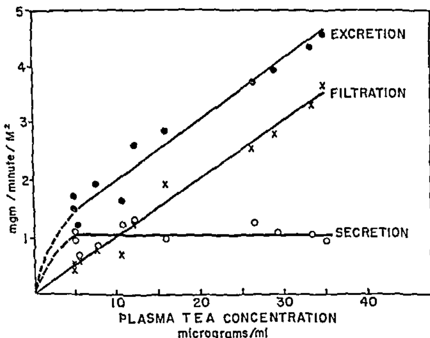


Figure 42. Relation between plasma tetraethylammonium concentration and its filtration, excretion, and tubular secretion in the dog kidney. Note that this quaternary ammonium base exhibits a secretory Tm in this species. From Rennick, Calhoon, Gandia, and Moe (40).

For instance, Farah and Rennick[44] have compared the uptake of TEA and PAH under a variety of conditions by kidney cortex slices of the dog. α-Ketoglutarate, succinate, and octanoate, while markedly inhibiting PAH uptake, had essentially no effect on that of TEA, confirming previous observations by the same authors

in the intact dog Study of the effects of cyanide, azide, sodium fluoride, iodoacetic acid, and 2,4 dinitrophenol showed that each of these inhibitors blocked the *in vitro* transport of both TEA and PAH Several other inhibitors, such as malonate fluoroacetate dehydroacetate, phlorizin, and Antimycin A (which the authors differentiate as ' Krebs' cycle inhibitors) showed the well known block of PAH uptake but were without effect on TEA They conclude that the transport mechanisms for TEA and PAH are different on the basis of their metabolic requirements, the TEA mechanism can continue to function after blocking the Krebs cycle by a number of inhibitors which depress PAH transport Therefore, they say "It is thus likely that the tetraethylommonium transport mechanism obtains its energy from a source other than the Krebs' cycle

Such a conclusion, based on studies with a group of inhibitors whose effects in fairly well organized tissue are not that specific would seem somewhat dangerous Phlorizin and Antimycin A cannot be considered primarily inhibitors of the Krebs cycle but rather affect sites in the terminal electron carrier system with secondary effects in the cycle As we will see later in our discussion of phlorizin, this inhibitor acts at a membrane and in mitochondria it results in a disruption of their osmoregulatory capacity, they swell in an isosmotic' environment and as a consequence the whole of their oxidative machinery is affected Thus the differing results obtained with a variety of inhibitors on PAH and TEA transport in slices must be interpreted with caution in terms of differing energy sources for the transport mechanism until it is carefully determined whether the inhibitors in their experiments were actually disrupting respiratory energy metabolism or blocking some non respiratory carrier system

Farah, Rennick, and Frazer[45] have studied the effect of a number of organic bases on tetraethylammonium transport in the dog renal cortex slices Some of these bases are themselves excreted by the tubules Priscoline, guanidine methylguanidine, piperidine, and NMN inhibited TF ... but in the concentrations used had little effect on ... PAH These findings afford ... l support for ... that TEA and ... rent mec

The demonstration *in vitro* that one base inhibits the uptake of another suggests that the authors are dealing with a situation where the several bases compete for the carrier system on the basis of substrate enzyme specificity It would be useful perhaps, to carry out kinetic studies relating inhibitor concentration (the second organic base) to reaction velocity (uptake of first organic base) in a Burke Lineweaver relation Even though such a study with slices or isolated mitochondria would be relatively crude, because one would be dealing with a multienzyme system conditioned by permeability factors and a complex organized steady state, nevertheless one might obtain some idea of the nature of the competitive interrelations and the relative affinities of the various bases for the carrier system

In another series of experiments on organic base uptake by slices, LeSher and Shideman[46] made observations on the metabolic aspects of Darstine transport They studied the effects of a variety of inhibitors, substrates, and PAH on Darstine uptake in dog kidney cortex slices They concluded that the Darstine transport system differs from both those for PAH and NMN because Darstine uptake is unaffected in the presence of PAH, NMN, or carinamide. Transport mechanisms for both PAH and Darstine were believed by the authors to derive a significant portion of their energy requirements from the Krebs' cycle Here again in the absence of respiratory measurements or careful kinetic studies conclusions about ' energy requirements have been drawn without the inclusion of permeability and surface carrier possibilities in the interpretation of the results

Baer, Paulson, Russo, and Beyer[47] have recently reported on the renal excretory characteristics of a new ganglionic blocking agent, 3 methylaminoisocamphane hydrochloride (Mecamylamine) This substance, also an organic base, exhibits an interesting excretory pattern that is exquisitely sensitive to urinary pH In clearance studies it was found that when the urine is acid the Mecamylamine is excreted by glomerular filtration and tubular excretion indeed, under these conditions the renal extraction is so complete that the Mecamylamine clearance measures effective renal plasma flow On the contrary, however, when the urine is made alkaline, either by infusion of sodium bicarbonate or the

administration of acetazoleamide, Mecamylamine is excreted by glomerular filtration and tubular reabsorption With the change from acid to alkaline urine the Mecamylamine clearance may drop from 200 to 3ml /min These characteristics are well seen in Figure 43 The tubular excretion of Mecamylamine in acid urines is unaffected by Benemid or PAH No data were presented in the paper on the effects of cyanine dye or other organic bases

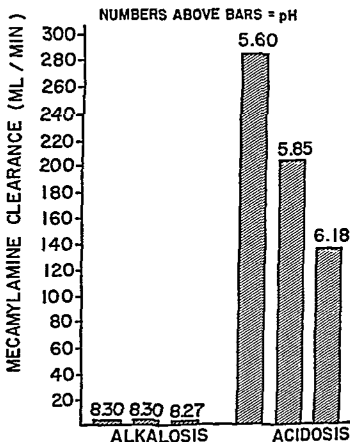


Figure 43 The effect of urinary pH on mecamylamine clearance in the dog In alkalosis mecamylamine is excreted by filtration and net tubular reabsorption in acidosis this pattern of excretion is changed to one of glomerular filtration with marked net tubular excretion Adapted from Baer, Poulson, Russo, and Beyer (47)

Baer *et al* attempted to explain the dramatic effect of urinary pH on Mecamylamine excretion in terms of the factors governing the distribution equilibrium of weak electrolytes between cells and their surrounding medium as described by Jacobs [48] It will be recalled in our previous discussion of the mechanism of ammonia excretion that these factors are The pH of the fluid medium the pKa of the base and the permeability of the cell membrane to the unionized lipid soluble species

The pKa of Mecamylamine is approximately 11 4 Thus at the prevailing pH of the tubular urine (approximately 7 0) one molecule of Mecamylamine in 25 000 exists as the base while at pH 8 ten molecules in 25 000 are present as the base Thus over this range of pH the change in quantity of unionized base is negligible yet over this same pH range the net tubular transport changes completely from excretion to reabsorption Therefore the striking change in the mode of excretion of Mecamylamine that occurs with changing urinary pH cannot be explained on the basis of a changing quantity of the unionized permeable species of the base

One wonders whether the total excretory process for Mecamylamine may involve a bidirectional tubular flux with one direction sensitive to changes in bicarbonate hydrogen ion system and the other not In this regard it would be instructive to see from stop flow studies the nephron site for Mecamylamine reabsorption in alkaline and secretion in acid urines One would also like to see experiments on the excretion pattern of Mecamylamine where urine pH is altered by a wide variety of means including potassium deficiency and respiratory change

The problem of organic base excretion and transport by the tubules has not yet been carried far enough to form any meaningful pattern in comparison to the studies of organic acid transport discussed in the foregoing section of this essay The list of organic bases transported by the tubules continues to grow thus recently there has been added histamine[49] and choline [50]

All these substances are strong bases and therefore like Mecamylamine their excretion cannot be explained in terms of of diffusion and permeability by the postulates of Jacobs which seem to govern the diffusion of the weak base ammonia and other

weak bases such as atabrine and quinine that have been studied by Orloff and Berliner[51] A continuation of studies with the inhibitors, unless they are quite specific in their effect at the various concentrations used, would appear to be more or less fruitless in the further approach to the organic base problem

It would perhaps be more fruitful to carry on experiments with molecular analogues of one or two of the bases whose transport has been carefully studied such as N methylnicotinamide or tetraethylammonium These studies could follow directions like those of Höber on the azo dyestuffs or Knoefel, Huang, and Despopoulos on benzoic and hippuric acid derivatives Knowing something about critical molecular configuration, one can then proceed to an understanding of the bonding between the base and its carrier in the cell membrane In this context one recalls the elegant studies of Rothstein[52] on glucose transport in yeast and those of Christensen[53] on the amino acid transfer mechanism in ascites tumor cells

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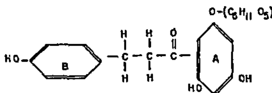
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VII

PHLORIZIN

IN 1885 VON MERING first noticed that the administration of phlorizin causes glycosuria, and for the purposes of the present discussion its biological significance stems from this observation Although it was used widely in nutritional balance studies to produce ' phlorizin diabetes we will be concerned here with the fact that it inhibits the biological transport of glucose and certain other sugars across the renal tubule, small intestine, and a number of other cell membranes Among these latter are those in kidney slices,[1] ascites tumor cells,[2] and erythrocytes[3 4] Thus its inhibition of sugar transport is a more general phenomenon than earlier suspected This fact will become more evident in the discussion to follow

Phlorizin is a glucoside of a polyhydroxyphenol Its hydrolysis yields dextrose and the aglucone phloretin It is found in the root bark of a number of fruit trees and was first isolated from the



Phlorizin

apple tree by de Konick in 1846 Because of its bitter taste, like quinine, this investigator administered it to persons with malaria However, it has no effect on this disease

The fact that phlorizin can completely block the tubular reabsorption of glucose in the kidney was revealed with some diffi

culty in three historically important studies The first of these was by Mayrs[5] who reasoned that if phlorizin were completely blocking the tubular absorption of glucose, the urine to plasma concentration ratio (U/P) of glucose after phlorizin should be the same as the simultaneous U/P of a no threshold substance At the time such substances were thought not to undergo tubular reabsorption, and for his purpose Mayrs chose sulfate This was not a bad choice because, though reabsorbed to a slight extent, at high plasma concentrations the reabsorptive rate becomes relatively insignificant in comparison to total excretion Unfortunately, however, Mayrs gave an inadequate subcutaneous dose of phlorizin (200 mg /Kg), and he observed similarity of glucose and sulfate U/P in only one experiment This led him to conclude that glucose reabsorption is not completely blocked by phlorizin

The second study, that of Poulsson,[6] came closer to proving the point He attempted to show that the glucose clearance could be used as a measure of glomerular filtration rate in the phlorizinized animal For this purpose he compared the 'phlorizinized' glucose U/P with the simultaneous creatinine U/P on the assumption that creatinine is neither reabsorbed nor excreted by the tubules He achieved glucose/creatinine U/P ratios after phlorizin as high as 0 96 and several between 0 80 and 0 90 On the basis of these studies, he concluded that phlorizin completely blocks the tubular reabsorption of glucose

Poulsson's results need only be qualified by the fact that in his time it was not yet clear whether in fact creatinine is excreted or reabsorbed by the tubules in some species However, this need only be a quantitative limitation on the interpretation qualitatively Poulsson was remarkably correct in his conclusions

The definitive study of Jolliffe, Shannon, and Smith[7] in 1932 showed the identity of simultaneous clearances of glucose, xylose, sucrose, and raffinose in the phlorizinized dog *(100 200 mg intravenously) and later, with the introduction* of the inulin clearance, it became clear that creatinine clearance of the dog does in fact, measure glomerular filtration rate Thus identity of glucose and creatinine U/P ratio finally established the fact that there is *essentially no glucose reabsorption by the tubule* after phlorizin in the dog

In addition to blocking glucose reabsorption in the kidney of the dog phlorizin produces a similar effect in man apes sheep chicken teleost fishes and the dogfish [8] Xylose which is reab sorbed to a lesser extent is also blocked in the dogfish dog rabbit and man [8] Piantoni[9] reports that vitamin C reabsorption is re duced by phlorizin However aside from this its effect seems to be remarkably confined to glucose among the normal constitu ents of the glomerular filtrate that are reabsorbed Phlorizin has no effect on the tubular reabsorption of chloride bicarbonate sodium amino acids or urea [8] Phosphate reabsorption is actually accelerated by phlorizin a phenomenon discussed earlier (Chap ter II) This effect probably results from the removal of glucose from some common element in the reabsorptive mechanisms of both glucose and phosphate

Besides the rather specific inhibition of glucose transport on the reabsorptive side of tubular function phlorizin is known to inhibit the tubular excretion of a number of organic acids Among these are phenol red in the chicken and man Diodrast in man and dog and creatinine in the dogfish chicken chimpanzie and man [8] It would seem at present however that these effects on tubular excretion are different from the inhibition of glucose re absorption for as we will see phlorizin itself is excreted by the tubules and probably competitively inhibits the transport of these other organic acids whose transport mechanism it shares

Kinetics of Phlorizin Inhibition of Glucose Reabsorption in Vivo

Before going on to an examination of the nature of the mech anism of the phlorizin inhibition of glucose transport it would seem best to look first at some simple kinetics of this process as it occurs in the intact animal For this purpose we will discuss some recent experiments on the dog performed in the author s laboratory [10]

The glycosuric doses of phlorizin usually given are relatively large in the order of 100 200 mg /Kg for dog and man Since phlorizin concentrations of the order of 10^{3} to 10^{5} M have been shown to produce inhibitory effects *in vitro* and since the phlor

izinizing" doses in dogs produce complete block of glucose re absorption, it was thought important to study the effects of smaller concentrations of phlorizin on the maximal capacity to transport glucose (Tm G) in the intact kidney In order to achieve a better steady state between transport mechanism and inhibitor,

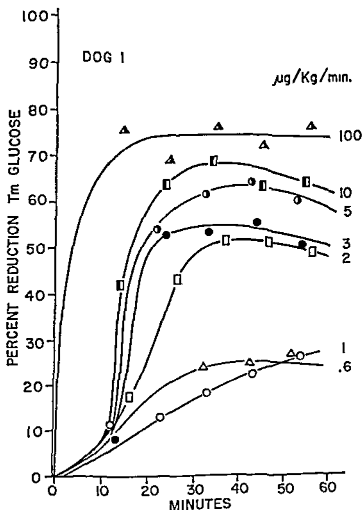


Figure 44 The effect of small graded doses of phlorizin given by continuous intravenous infusion on the Tm for glucose in the dog From Lotspeich and Woronkow (10)

it was decided to administer the phlorizin by continuous constant intravenous infusion rather than by single injection as is usually done in phlorizin studies

Accordingly in each experiment glucose (along with creatinine for filtration rate measurement) was infused in sufficient quantities to saturate the glucose reabsorptive mechanism After a long equilibration time (one and a half hours) three clearance periods were taken to establish the control Tm G Then phlorizin (along with the same concentration of glucose) was infused and successive periods were taken over a thirty to seventy minute

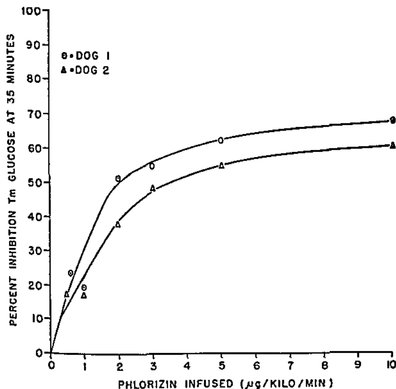


Figure 45 The relationship between rate of phlorizin infusion and the 35 minute inhibition of Tm glucose in two dogs For discussion see text From Lotspeich and Woronkow (10)

period and Tm G again measured Such studies were done in two dogs at phlorizin infusion rates that varied from 0 6 to 100 μg/Kg /min The results of a series of these experiments on one of the dogs is shown in Figure 44

At each dose level, even as low as 0 6 and 1 0 μg/Kg /min there was an increasing reduction of Tm G with time, and at rates of phlorizin infusion above 1 μg/Kg /min this inhibition of glucose reabsorption reached a plateau at around thirty five minutes The intensity of this inhibition and the rate of its attainment increased with dose, most markedly between 1 and 2 μg/Kg /min and less rapidly from 2 to 10 μg Even at 100 μg/Kg /min where a plateau of 75 per cent inhibition was very rapidly reached, the complete block of glucose reabsorption was not seen

If one accepts from these data that Tm G inhibition at any one infusion rate of phlorizin is essentially complete at 35 minutes and thus plots the 35 minute inhibition of Tm G against the rate of infusion of phlorizin, one obtains the dose response curves seen for two dogs in Figure 45 over the range of infusion rates up to 10 μg/Kg /min These curves, which have the charac teristics of an adsorption isotherm suggest that the infused phlor izin is bound with a very high degree of affinity to glucose carrier sites in the tubule cells and that the binding at varying rates of phlorizin infusion shows saturation kinetics

Since phlorizin itself is known to be excreted by glomerular filtration and tubular excretion,[11] this process itself might be expected to affect the rate of saturation of the glucose carrier sites with phlorizin that is, were the phlorizin itself not excreted actively, the local phlorizin concentration available to bind the carrier in the tubule might be greater during its constant in fusion Thus, although the general shape of the curve would be expected to be similar and to continue to look like an adsorption isotherm, its slope might be greater at any one phlorizin concen tration The excretion of phlorizin would only reduce, perhaps, the effective concentration for adsorption to the carrier at any one infusion rate

These data afford additional valuable information It is striking for instance, that Tm G should be reduced by phlorizin infusion rates as low as 0 6 and 1 0 μg/Kg /min This shows that phlorizin is a very potent glucose transport inhibitor, much more potent than hitherto fore realized A few simple calculations will illustrate this fact For example in the experiment where phlorizin was infused at 0 6 μg/Kg /min the total cumulative dose over thirty five minutes was 420 μg or 0 88 μMol The 20 Kg dog used in this experiment had a total body water of some 13 liters, assuming that this compartment is roughly 65 per cent of its body weight [12] If one makes the additional and strictly unjustified assumption, for the sake of an initial approximation that none of the phlorizin is metabolized or excreted and that it is evenly distributed in the body water one calculates its final body water concentration to be 5×10^{7} M

This concentration is considerably below the range of 10^{-3} to 10^{5} M found to affect sugar transport oxidative metabolism and mitochondrial permeability *in vitro (vide infra)* and also below those concentrations inhibiting glucose transport in the intestine Since phlorizin is not evenly distributed throughout the body water[13] and since it is excreted by the kidney it is unlikely that the 100 to 10,000 fold increase in concentration needed to reach these effective *in vitro* concentrations is actually attained at the phlorizin sensitive sites in the renal tubule One must conclude that in all likelihood the concentrations of phlorizin required to block glucose transport in the kidney *in vivo* are in the range of or even somewhat lower than those shown to have effects *in vitro*

In the experiments shown in Figure 44 it is evident from another line of reasoning that the affinity of phlorizin for the glucose carrier is of a very high order, much higher than glucose itself For instance, in one experiment phlorizin was infused at 20 μg/Kg /min This represents on a molar basis, $8\ 0 \times 10^{7}$ moles per minute In contrast to this, glucose was simultaneously being infused at $6\ 0 \times 10^{3}$ moles per minute Yet in this experiment glucose reabsorption dropped from a control rate of 297 mg /min to 92 and 94 mg /min by 10 minutes after the start of the infusion If one assumes (again for the sake of approxi

mation) that all the phlorizin given during the first 10 minutes was retained and evenly distributed through the extracellular fluid volume of this dog the phlorizin concentration in the extra cellular fluid would have been 2×10^{-6} M By actual measurement the extracellular glucose concentration was 4×10^{-2} M Yet glucose reabsorption with a constant filtered glucose load dropped about 70 per cent

Thus it is apparent that the effective concentrations of phlorizin that produce inhibition of Tm G *in vivo* are much lower than originally suspected and are in all likelihood within or below the effective concentration range *in vitro* This conclusion is in agreement with the findings of Jervis Johnson Sheff and Smyth[14] who found that the intestinal absorption of glucose is inhibited by phlorizin in very small concentrations (Figure 46)

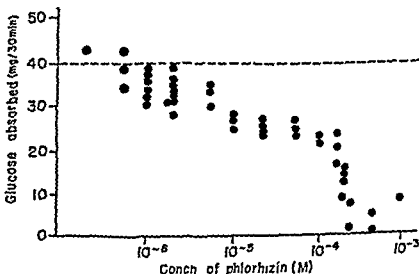


Figure 46 The effect of relatively small concentrations of phlorizin on glucose absorption in the small intestine. From Jervis Johnson, Sheff and Smyth (14)

At 10^{-6} M there is beginning inhibition which increases until at 10^{-3} M glucose transport is nearly 100 per cent blocked. The aglucone phloretin was much less inhibitory at comparable concentrations

These findings in kidney and intestine are very gratifying They emphasize first the remarkable sensitivity or better affinity, of the glucose carrier for phlorizin in both situations This fact agrees with the observations of Le Fevre[15] who studied glucose transport across the erythrocyte membrane and noted that phloretin which inhibits this process more than phlorizin has an affinity for the glucose carrier that is some 1800 times that of glucose itself

The Renal Excretion of Phlorizin and Phlorizin Glucuronide

Any complete account of the nature of the phlorizin inhibition of renal tubular transport must include a description of the excretion of phlorizin itself for this process must con

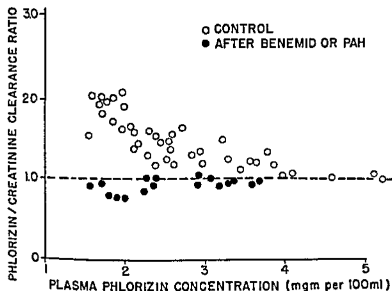


Figure 47 Data from the dog showing the net tubular excretion of phlorizin and its inhibition by *Benemid or PAH* Phlorizin-creatinine ratios above one indicate net tubular excretion Plasma phlorizin concentration was elevated stepwise by graded continuous intravenous infusion From Braun Whittaker and Lotspeich (11)

dition the way in which the phlorizin reaches the carrier sites where it produces its block.

Lambrechts[13] showed that phlorizin disappears from blood after its injection, believing it was metabolized. Using colored phlorizin compounds, he also showed that these are concentrated within the renal tubular cells and also appear in the urine. Recently Braun, Whittaker, and Lotspeich[11] studied the mechanism of excretion of phlorizin in the dog (with experiments on the chicken and the aglomerular fish, *Lophius americanus*, which substantiated their findings). These authors observed that phlorizin which is partially bound to plasma proteins, is both filtered at the glomeruli and excreted by the tubules. This tubular excretion is seen in Figure 47. The open circles, the phlorizin/creatinine clearance ratios, all lie above 1.00 below plasma phlorizin concentrations of about 4 mg. per 100 ml. The black circles in Figure 47 are from experiments during Benemid or PAH administration. The fact that the phlorizin/creatinine clearance ratios all group around 1.0 or a bit below it indicates that the tubular excretion of phlorizin is blocked by Benemid or PAH.

In the experiments illustrated in this figure the total quantity of phlorizin administered over the experimental period (approximately one hour) was 1 to 2 grams, an amount comparable to the usual glycosuric dose for a 20 kg. dog (100-200 mg./kg.). The fact that phlorizin is excreted by the tubules and that this process is blocked by Benemid probably explains the mechanism of the phlorizin block of the tubular excretion of phenol red, Diodrast, creatinine, etc. These substances, like phlorizin, are also inhibited in their tubular excretion by Benemid. Therefore, it appears that phlorizin is yet another member of the list of substances excreted by the same mechanism that excretes PAH, Diodrast, phenol red, etc. Its block of their tubular transport would appear to be on the basis of a straight competitive inhibition.

That the tubular excretion of phlorizin is in all probability not a major limiting factor in the phlorizin block of glucose reabsorption is evidenced by the fact that neither Benemid nor PAH have any effect on the reduction of Tm G during phlorizin infusion experiments of the type described above. This is true even where the phlorizin is infused at the lower rates of 2 to

5 μg/Kg/min Although the analytical method for phlorizin cannot detect it in plasma or urine at these low administration rates one would expect its tubular excretion to be high at such low plasma levels Thus from what we have seen the process whereby phlorizin blocks the tubular excretion of organic acids appears quite different from the one whereby it blocks sugar reabsorption

Formation and Excretion of Phlorizin Glucuronide

As can be seen in the first column of Figure 48 two strong phenol spots appear in the chromatogram of untreated urine one a larger spot with a high R_f which is phlorizin itself and another with a lower R_f On analysis this latter substance was found to be phlorizin glucuronide Its identification is shown in columns 2 and 3 of Figure 48 Elution and rechromatography of the spot again gave the single R_f spot (column 2) Elution and acid hydrolysis of this spot gave on rechromatography two spots corresponding to phlorizin and its aglucone phloretin And in

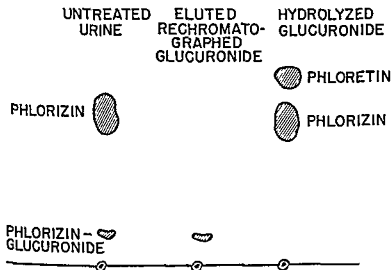
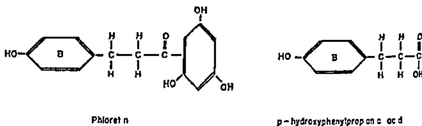


Figure 48 The chromatographic identification of phlorizin and glucuronide in the dog urine From Braun Whittaker and ?

addition the acid hydrolysate showed a positive Tollens reaction for glucuronides Thus during phlorizin infusion in the dog its glucuronide appears in plasma and urine

Booth, Jones, and De Eds[16] have recently shown that the rat injected with phlorizin or phloretin excretes not only the free aglucone but phloretin glucuronide as well In addition, they found in the urine another degradation product, namely p-hydroxyphenylpropionic acid This was shown to result from the cleavage of the two phenol rings of the phloretin molecule, leaving ring B with carbon side chain attached to form p hydroxy phenylpropionic acid according to the following reaction



In the dog the principal conjugated product appears to be phlorizin glucuronide on the basis of the evidence (Figure 48) Since it was not looked for at the time, it cannot be said whether p hydroxyphenylpropionic acid likewise appears in dog urine after phlorizin injection

The synthesis of glucuronides in the liver has been worked out in a beautiful series of experiments by Dutton and Storey[17 18 19] *These investigators found that* o-aminophenol glucuronide can be synthesized *in vitro* by mouse liver slices Liver homogenates were unable to form the glucuronide, but became able upon the addition of boiled liver juice The co-factor present in the liver juice was subsequently identified as uridine diphosphate glucuronic acid, and the overall reaction in glucuronide synthesis shown to be



ROH is the acceptor, in our case phlorizin, and UDP is uridine 5′ pyrophosphate Although attempts thus far by Whittaker[20] to synthesize phlorizin glucuronide by the Dutton and

Storey system have been unsuccessful there is no doubt that it is formed in the liver and perhaps also in the kidney

Jenner and Smyth[21] have recently shown that phlorizin glucuronide and probably some free phlorizin are excreted in the bile These studies nicely complement the present analogous observations on the urine The quantative excretion of phlorizin as the glucuronide in the bile is considerable and in Lambrecht s experiments where he thought phlorizin was being metabolized it was in fact probably being lost in the bile

Sperber[22] has shown that a number of glucuronides are excreted by the renal tubule in the chicken Since we had shown the presence of phlorizin glucuronide in the urine of the phlorizin infused dog it was of interest to examine the mechanism of excretion of the phlorizin glucuronide in the dog experiments described above Accordingly in each experiment phlorizin glucuronide was determined in plasma and urine by the same chromatographic phenol method used for phlorizin and its concentration expressed in terms of phlorizin equivalents

During phlorizin infusion phlorizin glucuronide/creatinine clearance ratios group with some scatter around 1 0 (open circles Figure 49) indicating that there is no reproducible tubular reabsorption or excretion of the glucuronide However when one gives Benemid all of the phlorizin glucuronide/creatinine clearance ratios fall well above 1 0 (black circles) over the whole range of phlorizin plasma levels studied This means that Benemid (or PAH) given during phlorizin infusion while inhibiting the tubular excretion of phlorizin itself (Figure 47) at the same time brings about a tubular excretion of phlorizin glucuronide

These observations could mean that both phlorizin and its glucuronide are capable of being excreted by the tubules but that during the infusion of phlorizin only the phlorizin itself is transported Now however when the phlorizin transport is blocked by Benemid or PAH the phlorizin glucuronide starts to be transported by a mechanism that is Benemid insensitive even though it is apparently phlorizin sensitive Whether phlorizin glucuronide is excreted by the tubules during infusion of pure phlorizin glucuronide is not known Such studies must await the ava '

ability of substantial quantities of this substance Such studies however, will be of great interest

One would suspect from the present experiments with Benemid and the studies of Sperber[22] that phlorizin glucuronide would be excreted by the tubules Such a study would be of great intrinsic interest to renal physiology In addition, it would be

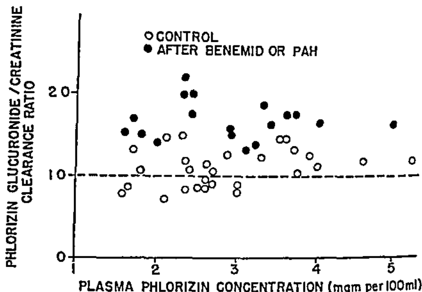


Figure 49 Experimental data supporting the conclusion that phlorizin glucuronide during phlorizin infusion is excreted by glomerular filtration without reproducible tubular reabsorption or excretion (open circles) However after the administration of Benemid or PAH phlorizin glucuronide excretion by the tubules appears to occur (black circles) This statement is based on the fact that after Benemid or PAH all of the phlorizin glucuronide/creatinine clearance ratios are well above one whereas before these agents these ratios group around one For discussion see text From Braun Whittaker and Lotspeich (11)

of interest to know whether phlorizin glucuronide is glycosuric and whether the process of glucuronide formation has anything to do with the mechanism of the phlorizin block of monosaccharide transport *in vivo* The fact that this process occurs in certain *in vitro* situations where glucuronide synthesis is absent[2,3,4] would speak against such a possibility However, these *in vitro* inhibi

tions by phlorizin show certain important differences from active monosaccharide transport *in vivo* and thus the role of the glucuronide of phlorizin *in vivo* still needs to be studied

Mechanism of Action of Phlorizin

Since the phlorizin inhibition of tubular excretion of phenol red Diodrast etc appears to be separate from the block in glucose transport the present discussion will be limited to a consideration of this latter phenomenon As will be evident the discussion will not be limited to studies with kidney or intestinal tissue but will include observations on a number of systems such as erythrocytes ascites tumor cells mitochondria and muscle

Ever since von Mering s discovery of the glycosuric effect of phlorizin physiologists have been interested in how it produces this effect That it is a block of tubular reabsorption of glucose and that its effect is quite specific at very low concentrations has been emphasized

In order to block a mechanism like glucose transport phlorizin would have to (a) compete with glucose for the carrier either competitively or non-competitively (b) change the physiochemical characteristics of the cell membrane and thereby reduce its permeability to glucose or (c) inhibit the energy producing reactions in the cell that drive the active glucose transport It is hard to conceive of a competitive inhibition between phlorizin and glucose in strict kinetic terms such as that between malonate and succinate The extreme sensitivity of glucose transport to phlorizin in kidney and intestine speaks rather of a non-competitive inhibition a conclusion strengthened by Le Fevre s work with erythrocytes

As we will see in the discussion to follow much evidence now points to a membrane site where phlorizin binds a glucose carrier with a high degree of affinity in a number of cell types The observable effects of phlorizin on energy metabolism appear to be secondary phenomena resulting from phlorizin induced changes in cell and mitochondrial membrane permeability and not directly related to the ultimate nature of the phlorizin block We will now trace historically the trail of evidence leading to this conclusion

Lundsgaard[23] thought that glucose transport both in kidney and intestine involves its phosphorylation and dephosphorylation. He therefore postulated that phlorizin inhibits these processes. However Lundsgaard himself rejected this idea in a paper published in 1935[24] in which he emphasized that the concentrations of phlorizin required to inhibit phosphorylations and dephosphorylations are too high. This conclusion is supported by the observations discussed above showing *in vivo* inhibitions of glucose transport at extremely low phlorizin concentrations in kidney and intestine. Furthermore it will be seen that *in vitro* effects of phlorizin appear in the concentration range (10^{-8} to 10^{-6}M) where there is no inhibition of hexokinase and where both renal and intestinal phosphatases are unaffected.[14, 25]

It was in 1947 that Shapiro[26] demonstrated an interference by phlorizin with both glycolysis and oxidative metabolism in minced rat kidney. Over the concentration range 10^{-5} to 10^{-3} M he found that phlorizin slowed the rate of lactate formation from glucose and the rate of oxidation of citrate and succinate. In addition he showed a depression of the phosphorylation of creatinine that accompanies oxidation in the kidney. The fact that succinate addition could restore creatinine phosphorylation to normal led Shapiro to conclude that phlorizin produces its block of glucose transport by inhibiting the synthesis of high energy phosphate compounds which are necessary to energize the process.

These findings were later extended and somewhat qualified by studies in the author's laboratory.[27] Using centrifuged homogenates of guinea pig kidney cortex fortified with Mg^{++}, ATP and substrates, it was found that phlorizin in a concentration range of 10^{-3} down to 10^{-5} M inhibits the oxidation not only of citrate but of all the substrates of the tricarboxylic acid cycle. In addition, it was found that the phlorizin depression of oxidation could be partially or completely overcome by an excess of any of the three adenine nucleotides ATP, ADP or AMP. This adenine nucleotide reversal, in the case of ADP, is seen in Figure 50. Here citrate was the substrate and ADP was not limiting the rate of respiration. Phlorizin 5×10^{-4} M reduced Qo_2 almost 50 per

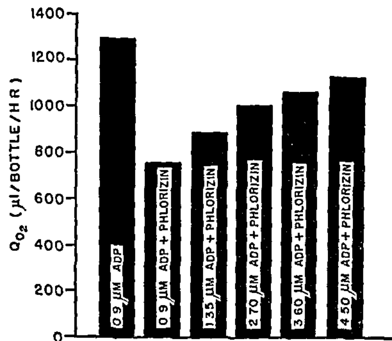


Figure 50 The adenine nucleotide reversal of phlorizin inhibition of citrate oxidation in guinea pig kidney homogenate *in vitro* Here is shown the relation between the level of adenosine diphosphate in the flask and the reversal of the oxidative inhibition produced by phlorizin in a concentration of 5 x 10^{-4} M ADP concentration was shown to be more than adequate to support maximal rate of respiration in the control flasks From Lotspeich (Unpublished observations)

cent The stepwise release of the phlorizin block with increasing ADP concentration is evident That this adenine nucleotide reversal can be seen with all three nucleotides is evident in Figure 51

It appeared at the time of these studies that phlorizin some how prevents the phosphorylation of ADP that accompanies oxi dation through the terminal respiratory electron carriers Chance and Williams had clearly shown[78] that respiration in mitochondria is limited by the supply of ADP and stops when it is all converted

to ATP A phlorizin block of the ADP ⇄ ATP conversion would then explain the general nature of the phlorizin effect on respiration Furthermore, this formulation would explain the fact that the addition of ADP (or a precursor) restores respiration to normal even in the presence of phlorizin As we will see, this explanation of the observed facts has undergone a change in the light of more recent experiments

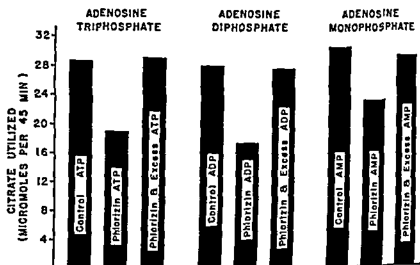


Figure 51 The adenine nucleotide reversal of phlorizin inhibition of citrate utilization in guinea pig kidney homogenate showing the phenomenon with adenosine triphosphate adenosine diphosphate and adenosine monophosphate In each situation the adenine nucleotide was present in more than adequate concentration to support maximal rate of respiration in the control flasks For discussion see text. From Lotspeich and Keller (27)

The phlorizin inhibition of oxidation and its adenine nucleotide reversal must be sharply differentiated from the inhibitory effect of 2,4-dinitrophenol (DNP) This substance which 'uncouples' oxidation from phosphorylation does not depress rate of respiration as does phlorizin, but typically accelerates it And furthermore the addition of excess adenine nucleotide does not reverse the effect of DNP as it does with phlorizin DNP at certain concentrations is probably an accelerator of adenosine tri

phosphatase (ATP ase) or acts as an ATP ase itself[20] Thus in its presence there is no impairment in the conversion of ADP→ ATP Rather the ATP, as rapidly as it is formed, is broken down to ADP and inorganic phosphate The net result is reduced uptake of phosphate and thus uncoupling However, respiration rate rather than being depressed is actually accelerated because an excess of ADP, so to speak is continually being regenerated within the system by the accelerated hydrolysis of ATP

The final sharp differentiation between the action of phlorizin and a typical uncoupler like DNP is seen in experiments (Figures 52 and 53) where phlorizin continues to inhibit citrate

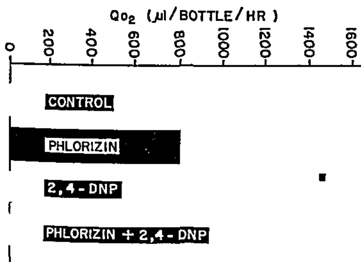


Figure 52 Experiment illustrating the fact that phlorizin produces its inhibition of citrate oxidation in guinea pig kidney homogenate *in vitro* even in the presence of strongly uncoupling' concentrations of 2 4-dinitrophenol From Lotspeich and Woronkow (10)

oxidation *in vitro* in the presence of DNP and where it continues to inhibit Tm G in the dog even though the animal was given DNP in an amount (10 mg /Kg) sufficient to depress PAH transport and to cause marked hyperventilation and hyperthermia

These remarks aimed to differentiate the effects of phlorizin from those of typical uncouplers are included here because there is a prevalent misconception that phlorizin produces its effects by uncoupling oxidation and phosphorylation and this idea is not strictly correct As we shall see below its effects on oxidation probably derive from a general membrane permeability alteration in mitochondria with secondary slowing of all the machinery of oxidation (including phosphorylation) that is housed in these organelles

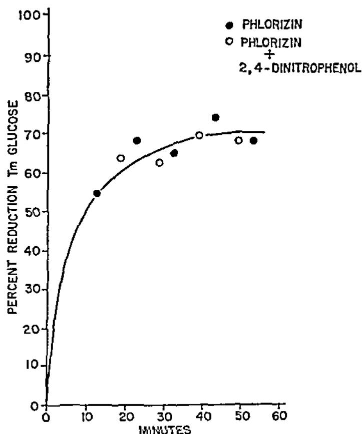


Figure 53 Experiment on the dog showing that phlorizin administered at a rate of 20 mg/kg/min produces the same inhibition of Tm glucose even after the injection of 2 4-dinitrophenol in a dose of 10 mg/kg For discussion see text From Lotspeich and Woronkow (10)

Effects of Phlorizin on Mitochondrial Metabolism and Permeability

Subsequent studies[30] with washed mitochondria have shown, in general, that the effects of phlorizin on the homogenate are likewise seen with isolated mitochondria Oxidizing α ketoglutarate with a P/O ratio of 2 8 to 3 6, mitochondrial respiration was inhibited by phlorizin concentrations similar to those having effects in the homogenate Also in mitochondria the adenine nucleotide reversal of the phlorizin block could be observed Therefore the studies with homogenates and Shapiro s work with the rat kidney mince, can best be explained in terms of an effect on mitochondria In view of the general nature of the phlorizin inhibition of respiration in homogenates and mitochondria, it was decided to see whether phlorizin has any general effect on mitochondrial structure which might alter their oxidative activities in the way that has been observed

For this study[31] it was decided to observe the effect of phlorizin on mitochondrial volume in isotonic sucrose Thyroid hormone and its metabolites have been shown to initiate isosmotic swelling of mitochondria[32] in sucrose media This phenomenon which has been shown to occur with a number of other agents such as detergents phosphate and carbon tetrachloride has been carefully studied by Tedeschi and Harris[33] and others If phlorizin were also to promote such isosmotic swelling of mitochondria and if adenine nucleotides were to prevent or reverse that swelling as they have been shown able to do in other instances,[34 35] then both the general inhibition of oxidation and the adenine nucleotide reversal would be explained That these suppositions were substantiated by experiment is shown in the data of Figure 54 *

Washed mitochondria were prepared by the method of Dounce[36] Mitochondrial volume was followed spectrophotometrically essentially according to the method of Tedeschi and Harris[33] and Tapley[37] Here changes in mitochondrial volume (as



determined by wet weight/dry weight ratio, direct microscopic visualization, or packed volume) are directly related to changes in transmission of light at 520 mμ Thus with no change in volume of mitochondria there is no change in density of the mitochondrial suspension at 520 mμ, but with swelling there is a decrease in density

In Figure 54 it can be seen that whereas there was no swelling in the control vessel in isotonic sucrose, there was a slow and progressive swelling of the mitochondria in the presence of phlorizin This was noticeable at a phlorizin concentration of 2×10^{-4} M, more marked at 5×10^{-4} M, and most evident at 10^{-3} M

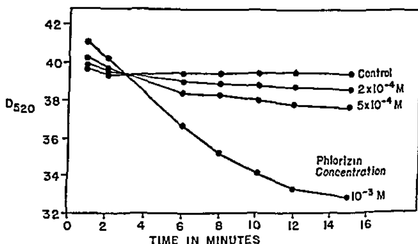


Figure 54 Experiments showing the phlorizin induced swelling of kidney mitochondria in isotonic sucrose Note that the rate of swelling is increased with the concentration of phlorizin From Keller and Lotspeich (31)

Tedeschi and Harris[33] have presented convincing evidence that the mitochondrial membrane is lipoidal in nature and that it is permeable to non electrolytes in direct proportion to their olive oil/water partition coefficient Davson and Danielli[38] have summarized the evidence supporting the concept that non-electrolytes diffuse through cell membranes *in direct proportion* to their lipoid solubility From Tedeschi and Harris s studies the mitochondrial membrane behaves like the cell membrane Thus the

phlorizin induced swelling of mitochondria may have general im plications concerning the action of phlorizin on the larger cell membrane

Since sucrose has almost no lipoid solubility and does not penetrate cells or mitochondria and since phlorizin causes mito chondria to swell in an isosmotic sucrose medium this phlorizin effect must mean that the mitochondria have become permeable to sucrose which then passes through their membrane As a re sult the interior becomes hyperosmotic to the medium water enters and swelling *occurs until osmotic equilibrium is re-estab* lished across the membrane

A number of other substances have been shown to initiate isosmotic swelling of mitochondria in a similar way among them inorganic phosphate [39] lysolecithin [40] and carbon tetrachloride [41] Recknagel and Malamed[41] have presented a clear analysis of the differences between isosmotic and hypo-osmotic swelling in mito chondria and have compared them to the same two types of hemo lysis The slow swelling of the mitochondria in the presence of phlorizin in isosmotic sucrose is characteristic of isosmotic swell ing and supports the conclusion that phlorizin is causing the mitochondrial membrane to become permeable to sucrose

Thus the phlorizin induced swelling of mitochondria appears to be part of a general phenomenon Since it is unlikely that phlorizin would act to increase the lipoid solubility of sucrose it seems more likely that pores are opened in the mitochondrial membrane allowing sucrose to enter Here then we first en counter in our discussion the idea that phlorizin may be affecting membrane permeability primarily rather than energy metabolism within the cell We shall return to this idea presently and will see that there is now a good deal of evidence to support it

Adenine Nucleotide Reversal of Phlorizin Induced Isosmotic Swelling of Mitochondria

Experiments were next performed to see whether any of the adenine nucleotides would prevent or reverse this phlorizin in duced swelling *It can be seen in Figure 55 that* ATP 5×10^{-3} M can completely prevent the phlorizin swelling phenomenon The addition of ATP to mitochondria that have already been swelling

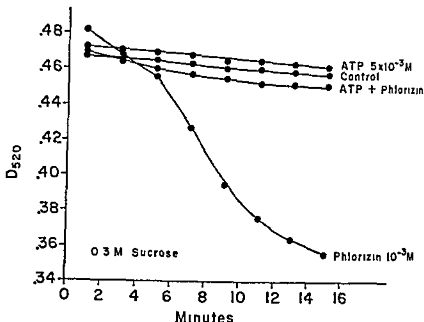


Figure 55 Experiments illustrating the fact that ATP protects kidney mitochondria from the swelling effect of phlorizin From Keller and Lotspeich (31)

for ten minutes or so can stop further swelling but not reverse it and shrink the mitochondria to their former volume (Figure 56)

In Figure 57 is seen a comparison of the effects of ATP, ADP, and AMP on the phlorizin swelling in mitochondria Here both ATP and ADP prevent the swelling, the ADP effect wearing off sooner than that of ATP In marked contrast, however, AMP (adenylic acid) is completely without protective effect

Raaflaub[34] first showed that ATP prevents the swelling of mitochondria Price, Fonnesu, and Davies[35] have studied mitochondrial swelling in hypotonic media and have also shown that ATP can prevent mitochondrial swelling in *hypotonic* solutions This remarkable adenine nucleotide effect, which can be shown also with ADP or AMP plus Mg^{++} ions, can even be shown to cause the extrusion of water from swollen mitochondria with their return to near control volume Our experiments were more like Tapley's[37] than these in the lack of complete return to normal

volume upon addition of ATP Prevention or reversal of swelling in the experiments of Price, Fonnesu, and Davies[35] correlated with ATP level in their mitochondria, and they stressed that phos phorylation must be proceeding for mitochondria to resist hypo-osmotic swelling An available supply of high energy phosphate added as ATP or ADP by the experimenter accomplishes the same thing

The fact that adenylic acid (AMP) did not protect the mitochondria in the phlorizin experiments (if they be looked at in

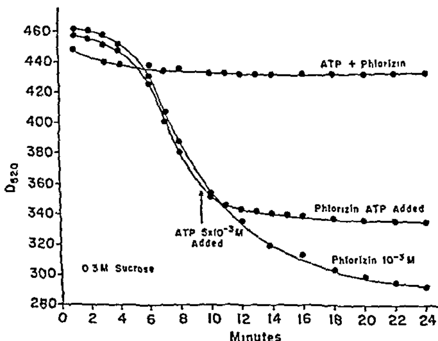


Figure 56 Experiment showing that the addition of ATP to mitochondria that have already started to swell under the influence of phlorizin can arrest that swelling phenomenon. The bottom curve illustrates the swelling of the mitochondria in the presence of phlorizin 10^{-3} M The middle curve in a parallel cuvette the arrest of the phlorizin swelling when ATP is a at the arrow The top curve shows the complete prevention of the phlo induced swelling when ATP is present in the cuvette from the begin From Keller and Lotspeich (31)

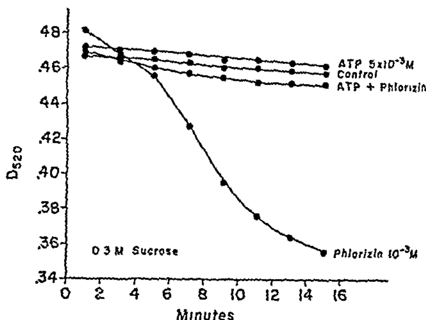


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bination of passive and active as in the experiments where oxidation and phosphorylation were actively proceeding Thus tonicity of the medium and presence or absence of oxidative phosphorylation obviously condition the nature of water and osmoregulatory activities of mitochondria and in turn then also condition the way in which drugs adenine nucleotides, and ions affect the overall process

Although the components of water and osmotic regulatory processes in mitochondria are complex and not yet well under stood, studies in this field should clearly state and qualify the exact conditions under which the experiments were performed. In addition we need much further work of the sort of Reck nagel and Malamed[41] clearly differentiating the nature of isos motic from hypo-osmotic and colloid osmotic swelling of mitochondria

These studies with mitochondrial swelling strongly suggest that the phlorizin inhibition of oxidative metabolism and the adenine nucleotide reversal phenomenon seen in kidney mince, homogenates and isolated respiring mitochondria are probably the result of fundamental alterations in the capacity of the mitochondria to regulate their water content in their normal cellular environment If Price Fonnesu and Davies[35] are correct, then phlorizin swollen but respiring mitochondria *in vivo*, in the pres ence of an adequate supply of intracellular adenine nucleotide, would be able to extrude water and restore their normal structure as the phlorizin is excreted in urine and bile This explanation of the capacity to recover from the effects of phlorizin in reason able concentrations agrees with the observation that kidneys from animals that have received single or infrequent glycosuric doses of phlorizin are remarkably normal looking under the microscope

On the other hand Nagai[42] has shown that kidneys from animals chronically treated with phlorizin show a depressed rate of oxidative phosphorylation and von Kossa quoted by Lusk,[43] found severe cloudy swelling of convoluted tubule cells from rabbits receiving large doses of phlorizin In view of the present belief that cloudy swelling of cells actually is the result of mitochondrial swelling these observations probably mean that phlori

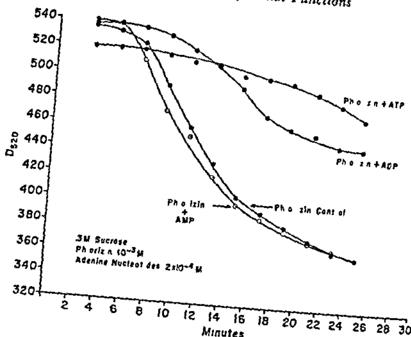


Figure 57 Experiments comparing the effect of ATP ADP and AMP on the phlorizin induced swelling of kidney mitochondria. ATP protects ADP also though less effectively while AMP has no protective effect on the phlorizin induced swelling For discussion see text From Keller and Lotspeich (31)

terms of the studies of Price *et al*) means that the AMP (which contains no high energy phosphate) could not be converted to ADP or ATP in the absence of substrate or Mg++ Our experiments[31] and those of Tapley,[37] in which ATP could prevent isosmotic swelling but not reverse it, differed in some important ways from those of Price *et al*[35] They were done in isotonic sucrose rather than a hypotonic medium containing substrate and Mg++ Thus although adenine nucleotides protect mitochondria from swelling under both isosmotic and hypo-osmotic conditions the two situations are obviously different in certain fundamental respects not well understood In addition the present studies with phlorizin[31] and those of Tapley[37] were performed under circumstances where there was no oxidation or phosphorylation going on Water movement was more likely passive rather than a com

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zin in sufficient single doses or in repeated doses can finally induce irreversible mitrochondrial swelling sufficient to cause recognizable cytological alteration These observations strengthen the concept that mitochondrial swelling and its prevention by adenine nucleotide explain the *in vitro* effects of phlorizin on oxidative metabolism

Since the mitochondrial membrane shares many of the structural characteristics of the larger cell membrane these findings with mitochondria raise the general question of a cell membrane site for phlorizin action A good deal of information from a variety of experimental work supports the idea that phlorizin acts at cell membranes and it is to this concept that we must now turn our attention

The Cell Membrane Theory of Phlorizin Inhibition

The experiments discussed above relating phlorizin concentration to inhibition of glucose transport in the kidney[10] and in the intestine[14] stressed that the glycoside produces its effects at extremely low concentrations Since selective cellular concentration of colored derivatives of phlorizin has been demonstrated in the kidney[13] and since the dose inhibition relationship between Tm G and phlorizin in the dog has the general characteristics of an adsorption isotherm it seems reasonable to suppose that phlorizin is being adsorbed or bound with a high degree of affinity to a monosaccharide carrier on the membrane rather than more diffusely localized inside the cell

This concept is supported by the recent experiments of Chinard Taylor Nolan and Enns[44] Using a single pass technique of rapid injection into one renal artery they constructed simultaneous curves of glucose and creatinine appearance rates in renal venous blood and were able to calculate from them mean transit time for the glucose through the glomeruli and across the renal tubules Thus they found to be of the order of 10 seconds They postulated from their data that tubular transport of glucose involves its combination with a carrier at the luminal membrane of the cell The fact that phlorizin blocks this process strengthens the conclusion that it binds a membrane glucose carrier

The penetration of a series of hexoses and pentoses into Ehrlich ascites tumor cells* and kidney cortex slices[1] where there is no active transport has been shown to be a reversible first order process governed by laws of diffusion Whether a carrier is also involved in glucose transport under these circumstances is not certain from the experiments If it is it is not concentrating the sugar inside the cell and thus no energy is required Even under these circumstances phlorizin in a concentration of 7×10^{-4} M caused an inhibition of 33 to 57 per cent (increasing at lower sugar concentrations) of this passive penetration of 3 methylglucose into Ehrlich ascites tumor cells at 10° C

Since the sugar penetration does not involve any requirement for energy under these conditions and since no phlorizin was found inside the cells one must conclude that the phlorizin was bound to the cell membrane and somehow decreased its permeability to the sugar or to its carrier bound form The authors point out that this phlorizin effect is non-competitive in nature a conclusion consistent with the observations discussed above on glucose transport inhibition in the dog kidney[10] and the erythrocyte [15]

Both Wilbrandt[3] and Le Fevre[4] have presented additional evidence supporting the concept that phlorizin and phloretin bind to a surface glucose carrier in the erythrocyte Rosenberg and Wilbrandt[45] have recently presented a theoretical analysis of the possible nature of this binding Studying the relative inhibitory potencies of phlorizin phloretin and a group of substituted phloretins they conceived the idea that one may view the phloretin molecule as a closed ring resembling a steroid when it is bound through a metal or hydrogen chelation with the membrane of the erythrocyte This steroid chelate concept gains support in the author s view from the fact that desoxycorticosterone glycoside is markedly glycosuric in the dog [46] Rosenberg and Wilbrandt then show in support of this concept that desoxycorticosterone or its glycoside are also inhibitors of glucose transport across the red cell membrane

This ingenious idea although unproven is most provocative and should lead to fruitful experimentation It leaves in doubt its applicability to the problem of phlorizin inhibition in kidney and intestine because in these situations phlorizin is a much more

potent inhibitor than its aglucone, phloretin. In the erythrocyte, however, the opposite is the case.

The concept of a membrane mechanism for phlorizin has recently received additional support from a quite different type of experiment done by Keller and Lotspeich.[47] Looking for a more general effect on which to test the membrane hypothesis, advantage was taken of the recent studies of Levine and his colleagues[48] who showed that insulin increases the galactose volume of distribution (space) in the eviscerate-nephrectomized dog. This observation has been generally interpreted to mean that insulin increases the permeability of muscle cells to glucose and thus facilitates its transfer into the cells. It was reasoned that if phlorizin acted generally at cell membranes, it might depress directly their permeability to sugars in the eviscerate animal or might antagonize the insulin-induced increase in sugar permeability.

To test this idea, female white rats were nephrectomized and made functionally eviscerate by the technique of Russell.[49] Galactose, which has the glucose configuration in its first three carbon atoms, is not metabolized in such a preparation and thus its true volume of distribution can be calculated. Initial experiments with this preparation confirmed the fact that insulin could increase the galactose space. At two hours this space, in terms of ml. per 100 grams body weight, was 28.3 in controls and 45 in those who had received insulin in a dose of 3.2 units subcutaneously at zero time. The next set of experiments revealed that phlorizin alone in doses as high as 14 mg./100 Gm. (a dose within the usual "glycosuric" range) has absolutely no effect on the galactose space (first two bars, Figure 58). However, in animals first given this dose of phlorizin and then given insulin thirty minutes later, the usual insulin expansion of the galactose space was not seen (last two bars, Figure 58). The difference in the galactose space between insulin-treated and phlorinized plus insulin-treated is highly significant ($P < 0.001$). There were nine animals in each group.

The meaning of this interesting observation is again not clear; however, that phlorizin somehow is able to block the action of insulin in increasing the permeability of certain cells to galactose does seem clear. We do not really understand the nature of the

insulin effect itself Insulin is a polypeptide of known amino acid structure which may combine with the lipoproteins of the cell membrane in such a way as to alter the spatial orientation of the molecules in that membrane and thus enlarge the ' pores through which the sugars can pass On the other hand, sugar uptake by cells has a carrier component, as we have seen Insulin and therefore phlorizin may act antagonistically on this carrier That phlorizin itself is capable of combining with cell surface proteins has recently been demonstrated in an interesting way by Rodriguez and Osler[50] They showed that the lysis of sensitized sheep erythrocytes by guinea pig complement is markedly inhibited by phlorizin in concentrations of 10^{-2} to 10^{-4} M

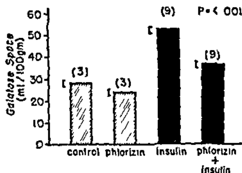


Figure 58 Experiments from eviscerate nephrectomized rats illustrating the way in which phlorizin blocks the insulin expansion of the galactose space For discussion see text From Keller and Lotspeich (47)

Thus the evidence from a number of different sources indicates that phlorizin attaches to a cell membrane site with great affinity and thereby blocks the transport of certain monosaccharides across it. The nature of this carrier binding is in all probability different in different cells For instance, the nature of the phlorizin (or phloretin) block of glucose transport across the erythrocyte is probably different from that in the proximal tubule cell of the kidney or the mucosal cell of the small intestine If for no other reason, the qualitative nature of the difference is evi

dent from the opposite effectiveness of phlorizin and phloretin in the two different situations

The concept of hydrogen or metal bonding of some kind be tween phlorizin and the carrier seems an attractive hypothesis as suggested by Rosenberg and Wilbrandt [45] With certain modifica tions this mechanism could apply to the kidney and intestine as well The phlorizin molecule contains several very reactive sites which could complex in several ways with reactive groups in the cell membrane or on its surface

In the author s laboratory it has been found that phlorizin forms a complex with borate Under these circumstances the characteristic ultraviolet absorption peak of phlorizin at 285 mμ disappears and a new peak of absorption appears at 325 mμ Since the 285 mμ absorption of phlorizin is probably due to its carbonyl group frequency it seems likely that the borate complex some how involves this group It may be a stabilization of the enol form on the carbonyl carbon similar to that caused by borate with a number of aromatic keto acids [51]

In addition Diedrich[5] has found that phlorizin forms a fluorescent chelate with aluminum This observation should pro- vide us with a very useful tool not only for the localization of phlorizin in cells but for studying the role of possible metal chelation in its mechanism of action The three hydroxyl groups for instance could link with NH_2 groups in the proteins of the membrane and thus alter the membrane structure In addition these hydroxyl groups are sites of possible esterification with a membrane component such as phosphate Being planar how ever they could not form a diester

The formation of hexose phosphate during glucose trans port has never been definitely ruled out Although phlorizin does not inhibit hexokinase[53] even in high concentrations there remains the possibility of formation of some phosphorylated form of glucose as the carrier complex which does not involve the conventional hexokinase ATP system The fact that it takes so few molecules of phlorizin to block the glucose carrier argues against such a possibility in view of the relatively large molecular concentration of phosphate in several forms in the cell Experi ments with O^{18} in the hydroxyl groups of phlorizin would pro-

vide valuable information about the types of bonding that could occur at these sites

The hydroxyl groups in the phlorizin molecule could also represent sites for internal bonding with the glucose side chain that is a bonding that would form a ring between substituted hydroxyl membrane carrier (such as metal hydrogen or phos phate) and some other reactive site on the molecule This was the sort of reasoning behind Rosenberg and Wilbrandt s[45] theory

If some such chelate were formed it might give a clue to the nature of the insulin permeability effect and its antagonism by phlorizin Is metal chelation involved in sugar transport the way it appears to be in amino acid transport? If so does such chelation affect the carrier without altering the lipoprotein struc ture of the membrane or does it alter the structure and thus its pore size or its permeability to the sugar? These questions it seems are fundamental to our understanding of this problem

Finally in this discussion we must refer to the interesting theory developed by Keston[54] to explain glucose transport and the mechanism of its phlorizin inhibition Keston has assigned a central role in sugar transport to the enzyme *mutarotase* This catalyzes the mutarotation equilibrium between α and β forms of D-glucose and a number of other monosaccharides Keston visua lizes that mutarotase is present on the luminal surface of the proximal tubule and as the glomerular filtrate passes down the tubule the glucose in it is acted upon by the enzyme During the mutarotation there is formed an intermediate state of D glucose that is neither the α nor β form Keston postulates that it is this intermediate low abundance form (the preferentially ab sorbed form PAF) that rapidly penetrates the membrane as fast as it appears in the enzymatically catalyzed reaction and passes to the point of lower intracellular concentration

Such a mechanism by setting up a concentration gradient of the PAF between lumen and cell would emphasize the minis cule requirements for energy in glucose transport In support of his theory Keston shows that all sugars that are actively trans ported are also mutarotase substrates while those that are not sub strates are not actively transported Those species that do not secrete sugar into the urine such as aglomerular fishes also do not

contain mutarotase in the kidney And finally phlorizin albeit in rather high concentrations inhibits mutarotase activity Kes ton also speculatively invokes mutarotase in the insulin facilita tion of glucose entrance into cells The fact that insulin does not cause a reproducible acceleration of glucose transport in the kidney may simply mean that the renal mutarotase is already max imally facilitated under normal circumstances or it may mean that insulin affects renal mutarotase less than that in muscle or lens where it exerts a facilitating effect Further studies with mutarotase are very desirable

Summary

Phlorizin the glucoside of a polyhydroxyphenol that is found in root bark of several fruit trees blocks the transport of glucose and a number of other monosaccharides in the kidney tubule small intestine erythrocyte and ascites tumor cell The experi mental evidence argues against a primary effect of this substance or its aglucone on energy metabolism and hence the energy supply for active transport Rather it appears to bind with great affinity a glucose carrier on the cell membrane The nature of this bind ing and the nature of the carrier remain obscure some form of chelation or hydrogen bonding between phlorizin and the cell surface seems a likely possibility Certain experiments link phlori zin and insulin as antagonists in the transport process phlorizin appearing to block the insulin facilitation of monosaccharide transfer into muscle cells in the eviscerate animal Whether these opposite effects of phlorizin and insulin represent antagonistic actions at one common carrier site remains to be seen

Inhibition of oxidative metabolism by phlorizin is apparently the result of changes in mitochondrial membrane structure which allows osmotic swelling with secondary alteration of the enzymatic machinery of oxidative metabolism housed in the mitochondria This formulation again supports the membrane theory of phlori zin action Although the exact nature of this membrane mecha nism is not clear at present, the experimental work thus far has led to a much clearer delineation of the phlorizin problem And in an understanding of this problem lies not only an explanation

of the mechanism of action of this interesting compound but, more importantly, an understanding of the fundamental biological process of glucose transport and its control by insulin in certain cells

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